

THE PRODUCTION OF DECIDUOMATA IN SPAYED IMMATURE RATS AFTER OESTRIN AND PROGESTIN TREATMENT¹

M. C. SHELESNYAK²

Department of Anatomy, College of Physicians and Surgeons, Columbia University

ONE FIGURE

The production of deciduomata in immature rats which have received gonadal stimulation with hypophyseal implants and basic extracts of pituitary has been reported (Shelesnyak, '31). Further investigation revealed the non-essentiality of initial anterior lobe implants, since animals which were given only basic extracts of pituitary or extracts of pregnancy urine, and threaded, formed deciduomes (Shelesnyak, '33 a, b). The role of the general somatic stimulator of pituitary substances as an aid in the production of deciduomata in prepubertal animals was not clarified. To test the necessity of such a factor, a series of spayed, immature rats was treated with oestrin and progestin to effect the hormonal phase essential to the production of a pro gravid uterus (Hisaw, Fevold, and Meyer, '30; Weichert, '28). The uteri of these animals were threaded and examined for decidual growths.

MATERIAL AND METHOD

Fifty-six immature female rats were spayed. The average age at castration was 19.2 days. Within 1 to 4 days after the removal of the ovaries, administration of oestrin (Amniotin, Squibb³) was begun. Two daily injections of oestrogenic

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² University Fellow in Medicine.

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hormone were made for 4 days. The total dosage per animal ranged from 4 to 100 rat units. At the end of the period of oestrin treatment, daily injections of oil solutions of extracts of hog corpora lutea (methods of Hisaw and Fevold, '30,⁴ and of Corner and Allen, '29) were initiated. The treatment with progestin was continued for 8 or 9 days. The total amounts of progestin ranged from 4 to 84 gm. equivalent of fresh tissue. No two animals (except in one instance where two animals were given 18 r.u. of oestrin and 18 gm. of corpora lutea) were given the same dosages of extracts. (Chart shows the dosages of individual cases.) On the fourth or fifth day of corpus luteum treatment, the uteri of animals were threaded with silk loops. The sutures were passed longitudinally through the lumen of the uterus after the described modification of the Long and Evans method (Shelesnyak, '33 b). On the fifth day after uterine stimulation, the animals were autopsied; the traumatized regions examined and fixed for microscopic study.

RESULTS

The administration of a great range of dosages, in order that a proper oestrin-progestin balance might be experimentally approximated, resulted in an ununiform distribution of responses. Of the complete series of 56 rats, 27 were negative. Twenty-three of the remaining 29 cases showed plus-minus ($\pm$) responses which consisted of small decidual areas demonstrable only by microscopic study. Six cases showed positive reactions (+) in which small but definite and discrete decidual growths were visible. The chart shows the responses of individual cases, and their distribution in respect to dosages of oestrin and progestin. The series is divided, in respect to hormonal dosages, into two groups. Area I on the chart includes all cases of animals which were injected with less than 25 rat units of oestrin and less than 32 gm.-equivalent of corpus luteum extract. Area II on the chart embodies all other cases. Sixty-six per cent (66.7 per cent) of the 27

⁴ Dr. H. Fevold kindly extracted a kilo of fresh hog corpora lutea for the author.

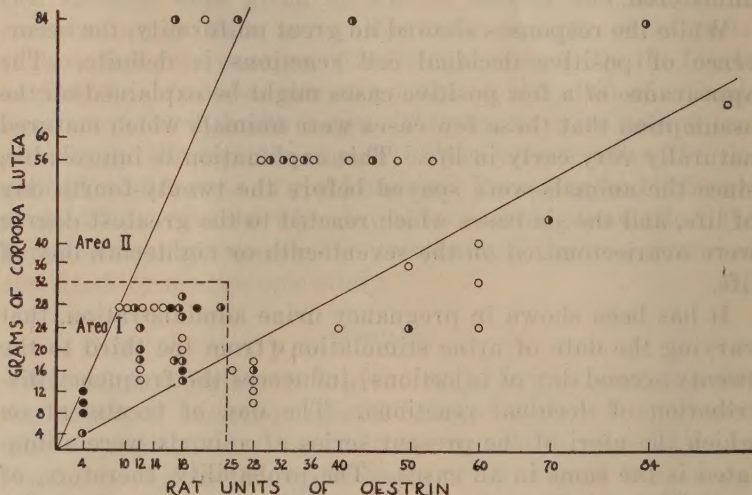
cases in area I showed decidual responses. The 6 positive (+) cases are confined to this group. Only 10 of the 28 cases in area II show any decidual reaction.

The experimental animals were spayed before puberty; and a decidual cell reaction was effected in the uterus, after a progestational endometrium was established with oestrin and progestin treatment. No gonadal or general somatic activators from pituitary substance or pregnancy urine were administered.

While the responses showed no great uniformity, the occurrence of positive decidual cell reactions is definite. The appearance of a few positive cases might be explained on the assumption that those few cases were animals which matured naturally very early in life. This explanation is improbable, since the animals were spayed before the twenty-fourth day of life, and the six cases which reacted to the greatest degree were ovariectomized on the seventeenth or eighteenth day of life.

It has been shown in pregnancy urine administration, that varying the date of urine stimulation (from the third to the twenty-second day of injections) influences the frequency distribution of decidual reactions. The day of treatment on which the uteri of the present series of animals were stimulated is the same in all cases. The probability, therefore, of a modifying influence caused by varying the data of uterine traumatization as in the P.U. treated rats, need not be considered. The most plausible explanation for non-uniformity of response probably concerns itself with the amounts of oestrin and progestin administered. It is to be noted that no two animals received the same amounts of extracts. If the dosages be causative in the distribution of reactions, then the region of optimal dosage will be demonstrable by an increased frequency of responses. This condition occurs, and the greatest concentration of decidual reactions is in a limited area (see chart). If all cases be divided into two classes: 1) those which received less than 25 rat units of oestrin and less than 32 gm.-equivalent of corpora lutea, and, 2) all other

animals, 27 cases will fall in class I and 29 cases in class II. From the graph it is evident that the greatest frequency of responses occurs in class I. In this group, 18 of the 27 cases (66.7 per cent) react. It is noteworthy that all the positive cases (6) fall within this group. In class II there are but eleven responses and these are plus-minus reactions. Such an analysis reveals the importance of quantities of materials in the elicitation of a decidual reaction.



Graph showing individual dosages of FSH-CL treated animals. The dosage of progestin in gm.-equivalent of fresh tissue is plotted as the ordinate; the dosage of oestrin in rat units is plotted as the abscissa. Clear circle denotes no response; solid black circle denotes positive response; half-black circle denotes plus-minus reaction.

When the CL/FSH (progestin/oestrin) of the extremes in area of class I are projected into area of class II, the class II cases fall roughly in equal numbers: 12 within and 17 without the projected area. Six plus-minus cases are found in area II when the CL/FSH is within the limits of the CL/FSH of class I, and five cases are found outside the limits. This data attaches a greater significance to the absolute amounts of material than to the quantitative relationship.

It cannot be denied that a hormonal balance is of importance. The work of Hisaw and Leonard ('30) and Leonard, Hisaw, and Fevold ('32) demonstrated that a balanced quantitative relationship between FSH and CL is necessary for the establishment of progestational modifications of the endometrium. They report that in the spayed adult rabbit, 10 r.u. of FSH, which inhibits the action of 1 rb.u. of corporin, did not suffice to suppress the activity of $1\frac{1}{2}$ rb.u. There is also much data presented by these workers, by Courier ('30), by Courier and Kehl ('30), Klein ('31) et al. that demonstrates the importance of considering the state of the animal (i.e., normal, oestral, dioestral, spayed, pregnant, pseudopregnant) in assaying the range of effective quantities of CL and FSH. From these data it is evident that the use of immature spayed animals in the present experiments permits quantitative standards for optimal activity, other than previously recorded.

In venturing an explanation of the lesser degree of responses in class II, aside from the lack of proper quantities of administered hormones, it may be suggested that the presence of oestrin in the corpora lutea extracts might reach an appreciably significant amount where the progestin is injected in large quantities, and/or that the excretion of surplus amounts of hormones, either one, or the other, or both, and at different rates, would influence the state.

Infantile rats which were given anterior pituitary implants and subsequently basic sheep pituitary extracts reacted to uterine stimulation to form deciduomata. Treatment with implants induces a general somatic change and initiates gonadal development (Smith and Engle, '27) and affects thyroid and adrenal conditions (Smith, '30). Since the effects of the hypophyseal implants and injections are somatic and gonadal, it cannot be assumed that the role of these substances in the production of deciduomata in the immature rat limits itself to an ovarian phase with the resultant discrete secretion of FSH and CL necessary for the establishment of a pro gravid uterus. Nor need it necessarily be true that the

pituitary extracts induced only an ovarian activity which resulted in a production of the proper amounts of oestrin and progesterin. It is logical to assume that an active fraction of pituitary substances, aside from the ovarian activator, may have aided in the establishment of a functional endometrium. By the direct application of oestrin and progesterin to spayed immature rats, pituitary substances need not be used to induce gonadal secretion of FSH and CL. The general somatic stimulatory effects of pituitary material are avoided. This endocrine condition of the infantile uterus is similar to the condition required by the adult in order that a progravid endometrium may develop. The induction of positive decidual reactions in the immature rat after this FSH-CL treatment shows that the infantile uterus can produce certain physiological phenomena (to wit, deciduomata) without the influences of general somatic development. The threshold of endometrial activation necessary for the production of deciduomatous formations in the infantile uterus consists of a di-phasic hormonal state: oestrin and progesterin.

It may be concluded that the uterus of the immature rat can respond to specific physiological treatment (FSH and CL) and form deciduomes, without the introduction of a general somatic or gonadal development.

SUMMARY

Spayed infantile rats were treated with oestrin and progesterin, respectively. During the period of progesterin treatment, the uterine mucosa was stimulated in an effort to provoke deciduomatous growths. The range of injected materials was large (FSH, 4 to 100 r.u.; CL, 4 to 84 gm.). Positive responses occurred, and were limited to animals which received lower dosages of hormones. The uterine response was dependent upon quantities of oestrin and progesterin, and was effected without the aid of pituitary stimulation.

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THE EFFECT OF UNILATERAL SECTION OF THE PERONEAL NERVE OF THE ALBINO RAT ON THE NUMBER OF MYELINATED FIBERS IN THE INTACT NERVE OF THE OPPOSITE SIDE

K. TAMAKI

The Wistar Institute of Anatomy and Biology

There are, in the literature, incidental observations to the effect that injury to a given nerve on one side of the body is followed by changes in the corresponding nerve on the opposite side. These observations will be considered later.

In view of such findings, it was decided to determine specifically what happened in the intact peroneus nerve of the rat when the corresponding nerve on the opposite side of the body was sectioned.

This problem was suggested to me by Dr. H. H. Donaldson, and the study of it was carried out under his direction.

There were two series of observations made. Series 1 was directed to the determination of the number of myelinated fibers present in the intact nerve after an interval of 42 days following the operation, and series 2 for the determination of the normal increase in the number of myelinated fibers in the peroneal nerve during the same interval of time, in unoperated rats of like age.

MATERIAL AND METHOD, SERIES 1

Nineteen albino rats—9 males and 10 females—were used for this first study. The rats were from the Experimental Strain of The Wistar Institute Colony. All were normal.

The ages of the animals at operation ranged from 155 to 175 days. The other related data are given in tables 1 and 2.

OPERATION

Under ether, the hair was removed from the popliteal space with the aid of a sodium sulphide solution. An incision about 2 cm. long was then made above the peroneal nerve, and the surrounding tissues carefully withdrawn. Silk threads were then passed beneath the nerve, and tied at each end of about 1 cm. of nerve to a light bristle, bent in a U form. This to keep the strip of nerve normally extended.

As thus extended, the strip was removed, the proximal end being indicated by a longer thread.

The wound was closed by one or two skin sutures. No antiseptics were used. Recovery was rapid. The inversion of the foot and the flexion of the toes caused by the operation had almost disappeared by the tenth day, and at 42 days after the operation all the rats were alive and in good order. They were then killed with ether, and a similar strip removed from the intact nerve by the method just described.

The operation in ten rats was on the right side and in nine rats on the left side. This was done in order to avoid any asymmetry in the number of fibers according to side. However, there was already good evidence (Greenman, '17) that such symmetry did not obtain. At autopsy no pathological conditions were found save the atrophy of the muscles of the foot and leg on the side of the operation.

The strip of nerve to be used for study was fixed in a 1 per cent osmic acid solution, embedded in paraffin, and sectioned at 10 μ .

As the number of fibers in a nerve tends to increase distally (Dunn, '02; Donaldson, '03; Greenman, '13), sections were taken always as near as possible to the central end of the strip.

Enumeration of fibers was made by the photographic method described by Hardesty (1899) and modified by McCrady ('33). A negative picture on bromide paper, at a

magnification of 325 diameters, was used. The number of fibers was recorded by pricking a hole in each fiber on the photographic sheet with a needle attached to an automatic counter (Hardesty, 1899). For control, the corresponding section was kept available under an immersion lens. As the negative print is a reversed image of the original section, a prism was attached to the ocular (McCrady, '33), by which the image in the microscope was brought into agreement with that on the print.

RESULTS. SERIES 1 (tables 1 and 2)

The number of myelinated fibers was counted in sections from both the normal and the test nerves in nineteen rats. The normal nerve was the one removed at the time of operation, while the test nerve was that removed 42 days after the operation.

The data given for two groups in each sex, in tables 1 and 2, show what was found.

TABLE 1 (Series 1)

Males. Number of fibers in the peroneal nerve, a) at age of operation; b) in the peroneal nerve of the opposite side, 42 days later

GROUP A, 4 CASES	AGE, DAYS	ED. WT., GM.	ED. LGTH., MM.	NUMBER OF FIBERS	PERCENTAGE DEVIATION OF TESTS
(a)	162	351	...	1963	
Test (b)	204	380	241	2006	+ 2.1
GROUP C, 5 CASES					
(a)	167	308	...	2005	
Test (b)	209	332	226	2020	+ 0.7
Average deviation of tests,					+ 1.4

It appears, from tables 1 and 2, that in each of the four groups the number of fibers in the test nerve is, after 42 days, greater. The excess, however, is slight, being on the average 1.4 per cent for the males and 0.5 per cent for the females. Thus, 42 days after the operation, the number of fibers in the test nerves has increased, on the average for both sexes, 0.9 per cent.

TABLE 2 (Series 1)

Females. Number of fibers in the peroneal nerve, a) at the age of operation; b) in the peroneal nerve of the opposite side, 42 days later

GROUP B, 5 CASES	AGE, DAYS	BD. WT., GM.	BD. LGTH., MM.	NUMBER OF FIBERS	PERCENTAGE DEVIATION OF TESTS
(a)	164	233	...	2025	
Test (b)	205	241	204	2031	+ 0.3
GROUP D 5 CASES					
(a)	167	231	...	1948	
Test (b)	209	251	207	1962	+ 0.7
Average deviation of tests,					+ 0.5

To further analyze the relations between the numbers of fibers in the two contrasted nerves, we proceed as follows: Neglecting sex, the nineteen individual cases are tabulated in three groups: Namely, 5 rats in which there was a loss of fibers in the tests, 6 rats in which there was little difference, and 8 rats in which there was a definite gain of fibers in the tests.

Table 3 shows the percentage deviations for these groups of contralateral test nerves.

TABLE 3

Showing the percentage values for the deviations in each of three groups. Based on data for series 1

	<i>Deviation in test nerve</i>
Group A. 5 cases: loss of fibers	-4.7 per cent
Group B. 6 cases: little change	+0.8 per cent
Group C. 8 cases: gain of fibers	+4.7 per cent
Weighted average	+0.9 per cent

It appears, from this analysis, that the response of the test nerve to section of the peroneal nerve on one side is various. In some cases (group A) there is a distinct loss of fibers, while in group C the fibers still increase, though, as will be shown when the data for the second series are discussed, this increase is less than normal. It may be noted here that the error of observation has been found to be about 1 per cent.

To interpret these results, it is necessary to determine the increase in the number of myelinated fibers during 42 days in the intact rats of like ages. For this purpose a second series of observations was made.

SERIES 2 (tables 4 and 5)

The purpose of these observations was to determine the normal increase in the number of myelinated fibers in the peroneal nerve of intact albino rats at the age and for the interval indicated in series 1.

For this purpose fifteen rats were used: Two younger groups, consisting of 3 males and 4 females, and having an average age of 163 days, and two older groups of 4 males and 4 females, respectively, having an average age of 205 days.

TABLE 4 (Series 2)

Males. Number of fibers in the right and the left intact peroneal nerves—at 163 days and at 42 days later

	AGE, DAYS	BD.WT., GM.	BD.LGTH., MM.	NUMBER OF FIBERS		PERCENTAGE DIFFERENCE BY SIDE
				Right	Left	
Younger group G, 3 cases	163	338	236	2027	2031	L + 0.2
Older group E, 3 cases	205	331	232	2168	2152	R + 0.7
Gain				141	121	
Mean				131		
In per cent				+ 6.9	+ 5.9	
Mean				+ 6.4 per cent		

All these rats were killed at approximately the same time, and sections from both the right and left nerves of each animal were prepared. All the subsequent procedures were similar to those used for series 1.

The results, in terms of the number of myelinated fibers in the right and left nerves, are given in tables 4 and 5, and there are also entered in these tables several computations.

The figures in table 4, for the males, show that the number of fibers in the right and left nerves is similar, both in the

younger and older groups, for differences under 1 per cent are hardly significant.

Contrasting the numbers for the younger group with those for the group 42 days older, it is seen that in the males there is a mean gain of 131 fibers, which amounts to 6.4 per cent, or about 3 fibers per day.

TABLE 5 (Series 2)

*Females. Number of fibers in the right and in the left intact peroneal nerves—
at 163 days and at 42 days later*

	AGE, DAYS	BD.WT., GM.	BD.LGTH., MM.	NUMBER OF FIBERS		PERCENTAGE DIFFERENCE BY SIDE
				Right	Left	
Younger group H, 4 cases	163	233	209	2064	2062	R + 0.1
Older group F, 4 cases	205	227	212	2171	2177	L + 0.3
Gain				107	115	
Mean				111		
In per cent				+ 5.2	+ 5.6	
Mean				+ 5.4 per cent		

Similarly, in table 5, for the females, there is even less difference in the number of fibers according to side. In this instance the mean increase in the number of fibers is 111 in 42 days. This amounts to an increase of 5.4 per cent, or about 2.6 fibers per day.

Thus, in respect of gain of fibers the two sexes are behaving in nearly the same way.

Comparing the data for both series 1 and 2, we have the relations shown in table 6.

TABLE 6

*Showing the percentage increase in the number of fibers in the intact peroneal nerve after 42 days, 1) in rats operated on one side and,
2) in rats not operated*

1. Rats operated		2. Rats not operated
Males	1.4 per cent	6.4 per cent
Females	0.5 per cent	5.4 per cent

From these data it is evident that section of peroneal nerve prevents the contralateral intact nerve from showing the

normal increase in the number of fibers during 42 days after operation.

When the detailed records comprising the several groups are examined for the possible influence of body weight or body length at like ages, it is found that only in the case of body length is there an indication of a slight increase in the number of fibers. This influence of body length may, however, be neglected here, in view of the dominating influence of age.

Touching the nature of the changes with age (series 2) in the fiber content of the normal peroneal nerve and the changes in fiber content in the test nerve after contralateral section (series 1), only probable explanations can be offered.

It is to be remembered that myelinated fibers alone are here considered. In the normal peroneal nerve the more distal sections show a greater number of fibers than appear in the more central sections (Greenman, '13). This peripheral increase in the number of myelinated fibers might be due to the more complete myelination of the axons already present in the distal portion of the nerve, or to the appearance of a gradually increasing number of fiber branches which are myelinated. In view of the observations of Dunn ('09), of Hardesty ('00), and of many others, the latter view, namely, an increase in the number of fiber branches, seems the more acceptable.

In these terms, therefore, the distal increase in the number of fibers and the increase with age (series 2) is probably due to an increase in the number of myelinated fiber branches.

When the intact (test) nerve responds to a contralateral section by changes in the number of myelinated fibers, it does so, most probably, through the fiber branches above mentioned. As is shown in table 3, the formation of these branches may be retarded or nearly completely stopped—or finally a certain proportion of them either lose their sheaths or atrophy completely—a range of response quite familiar in studies on the degeneration of neurons. Unfortunately, at the moment it cannot be stated whether the responding

branches belong to afferent or efferent fibers. Whether the responsive disturbances in the fiber branches cause, in this case, recognizable changes in the cells of origin has still to be determined.

As to the means of causing the changes in the fibers of the test nerves, there appear to be two possibilities: It may be brought about either, 1) through a disturbance of histological connections of the respective cells of origin on both sides, or, 2) through a humoral influence, i.e., by substances arising from the affected neurons on the side of the section and acting specifically on the corresponding neurons of the other side. At the present moment the humoral influence seems to be the more plausible.

DISCUSSION

In the opening paragraph of this paper, it was stated that there were other observations on the contralateral effects of a lesion on one side.

When the motor nerves of the lumbar plexus of a frog were torn away from the cord on one side and allowed to degenerate completely, it was found by Dunn ('09) that the motor fibers to the thigh of the opposite side were reduced in number by about 17 per cent.

Greenman ('13) estimated the loss of fibers in the peroneal nerve, after a contralateral section, as 16 per cent in one comparison and 12.3 per cent in another. The interval between operation and examination was, in these cases, something over 100 days. Doctor Greenman recognized the significance of this response, and began a further study of it, but was unfortunately prevented from completing the work.

Turning now to the afferent system, Nittono ('23) observed, after section on one side of either the fifth nerve or one of its branches, evidences of disturbance in both the ganglion cells and fibers of the intact side.

And Koester ('03), after section of the vagus below the ganglion jugulare (in the rabbit), found some degeneration of both cells and fibers on the intact side of the operated ani-

mal. He was inclined, however, to consider this condition on the intact side as normal, in the sense of Sigmund Mayer's report (1881). However, Duncan ('30), after a careful study, finds in the peripheral nerves of the rat, under normal conditions, less than 1 per cent of degenerated fibers, and often much less.

The observations here reported have, then, experimental support from previous observers.

There is, moreover, a considerable body of clinical observations touching this problem of contralateral effects of injury on one side, but the discussion of these reports belongs elsewhere.

From the foregoing observations the following conclusions are reached.

CONCLUSIONS

In the peroneal nerve of the normal albino rat, the number of myelinated fibers at a given level, in rats 163 days old, increases in the course of 42 days by 6.4 per cent in the males and by 5.4 per cent in the females.

In albino rats with the peroneus nerve sectioned on one side there is, in a series of nineteen, an average increase in the number of myelinated fibers in the intact test nerve amounting to only 0.9 per cent. In one group of these there is a loss of fibers; in another group, a cessation of increase, and in the third group an increase which is retarded.

Section of the peroneus nerve of one side thus produces changes in the contralateral nerve, expressed as a loss of fibers or a retardation in their production with age.

The fibers so affected are thought to be myelinated branches of the main fibers forming the nerve.

It seems probable that these changes induced in the contralateral nerve are due to humoral stimuli represented by substances arising in the damaged neurons of the sectioned nerve.

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ON THE SECTIONAL AREAS AND VOLUMES OF THE
LARGEST MOTOR CELLS AND THEIR NUCLEI
IN THE LUMBAR CORD OF THE ALBINO
RAT—ACCORDING TO SEX

K. TAMAKI

The Wistar Institute of Anatomy and Biology

Recently, K. Ide ('30) noted, in relation to age, a sex difference in the sectional areas of the largest fibers in the ventral roots of the 4" and 5" lumbar nerves of the albino rat, the sectional areas for the female being the larger. Naturally, the question arose as to whether a similar sex difference existed for the sectional areas of the corresponding motor cells in the spinal cord.

My study on this subject was suggested by Doctor Donaldson and was carried out under his direction.

MATERIAL

Albino rats from the 'experimental strain' of the animal colony at The Wistar Institute were used. These rats showed no pathological defects.

To eliminate the age factor, the members of each pair (often brother and sister) were of approximately like age.

Ten males and 10 females, the pairs ranging in age from 607 to 687 days, were originally selected for study. These were divided into two groups on the basis of body length. Subsequently, a third lot, the pairs ranging in age from 437 to 627 days, was added. This last lot contained 6 males and 6 females. It was found necessary later to exclude the preparations from 1 male in the second group and 1 male in the third group. As a consequence, this study is based on observations made on 14 males and 16 females.

TECHNIQUE

The rats were etherized and records made for the sex, age, body weight, and body length. The viscera were then removed, to facilitate further dissection. The cord was exposed and the nerve roots cut away from all but the 4" and 5" lumbar segments. These segments were then separated from the rest of the cord and the specimen was fixed in Susa's solution for 6 hours, embedded in paraffin, sectioned at 20μ , mounted serially, and stained with thionin blue. Care was taken to use a uniform technique throughout.

MEASUREMENTS OF CELLS AND NUCLEI

The measurements were made with an oil immersion lens and a wheel eye-piece micrometer—one division on the wheel being equal to 1μ in the field.

Two diameters were measured in both the cells and the nuclei. One diameter was the longer, and the other, the shorter, was measured at right angles to it. In each case the line of measurement passed through the nucleolus.

The sections were cut from the cephalic end of the segment caudad, and the slides numbered accordingly. The average number of slides obtained from each specimen was twelve. Usually five slides from the middle of the series were used. Fifty measurements were made on each specimen, comprising ten on each slide. The sections on the slide were chosen at regular intervals, and only one cell and its nucleus was measured in a given section.

The aim was to select the largest cells in the ventro-lateral group. Sometimes larger cells appeared in other parts of the ventral horn, but these were neglected. Only those cells showing a nucleolus were measured.

THE INITIAL RESULTS

The initial results are shown in tables 1 and 2. Table 1 contains the data for the cells and table 2 those for the nuclei.

In table 1, for the cells, the data for the averages in each sex are entered by groups. These data are for age in days; for body weight in grams; for body length in millimeters;

TABLE 1

Data for the determination of the areas in μ^2 and of the volumes in μ^3 of the largest motor cells in the 4" and 5" lumbar segments—albino rat. Average values

Group 1. Averages							
	AGE, DAYS	BODY WT., GM.	BDY. LGTH., MM.	DIAMETERS, μ		AREAS, SQ. μ πab	VOLUME μ^3 $4/3\pi a^2b$
				Long	Short		
5♂	647	341	234	52.6	39.9	1645	44068
5♀	642	246	207	50.7	39.0	1552	40273
Group 2. Averages							
4♂	646	422	245	50.3	40.5	1591	43318
5♀	649	297	217	50.9	38.0	1518	38409
Group 3. Averages							
5♂	561	398	238	53.2	39.3	1650	43230
6♀	538	282	212	53.1	38.3	1597	40809

TABLE 2

Data for the determination of the areas in μ^2 and of the volumes in μ^3 of the nuclei in the largest motor cells—already measured—in 4" and 5" lumbar segments—albino rat. Average values

Group 1						
	BODY WT., GM.	BDY. LGTH., MM.	DIAMETERS, μ		AREAS, SQ. μ	VOLUME μ^3
			Short	Long		
5♂	341	234	19.9	17.4	271.9	3125
5♀	246	207	19.6	17.5	269.5	3120
Group 2						
4♂	422	245	19.6	17.3	266.5	3079
5♀	297	217	19.6	17.0	262.2	2955
Group 3						
5♂	398	238	19.0	16.4	244.8	2750
6♀	282	212	18.9	16.5	244.7	2692

for diameters, long and short, in μ ; for areas in μ^2 , and for volume in μ^3 . The areas were determined by the formula for the ellipse— πab —in which 'a' is the half-diameter, short, and

'b' the half-diameter, long. The volumes were determined by the formula for the ellipsoid— $4/3\pi a^2b$ —in which the half-diameter, short, is taken twice and hence appears as a^2 .

By the aid of these data, the relations of the areas and volumes of the largest motor cells—according to sex—can be determined in several ways.

In table 2—for the nuclei—the data for age, body weight, and body length are, of course, the same as in table 1 for the cells. Since the data on age are not used in the further computations, they are omitted from table 2. The average values of the data for the three groups are alone given.¹

It is possible to make a further comparison between the areas of the cells and the areas of the cross section of the fibers coming from them by the use of data furnished by K. Ide.

In table 3 are given the data for the areas of the cross sections of the largest nerve fibers in the ventral roots of the 4" and 5" lumbar nerves, at like ages. The records are from tables 5 and 5a in K. Ide's study of 1930.

From Ide's table 5 we obtain the data for these fiber areas in both nerves of both sex groups. From Ide's table 5a the data are for the 5" lumbar nerve only. As the previous determinations on the cells and their nuclei have been made from both the 4" and 5" lumbar segments combined, the areas of the corresponding fibers should be equally represented in any comparison between the two sets of data. For this reason the necessary determinations for the sex relations in the areas of the fibers in the missing ventral root of the 4" lumbar nerve (table 5a) have been computed on the assumption that they would be related to the observed data for the 5" lumbar—as are those for the 4" and 5" lumbar in table 5.

¹The diameters of both cells and nuclei as recorded in tables 1 and 2 are larger than was expected—from previous studies. In previous studies the Bouin fixation had been used, while in the present instance Susa fixation was employed. A special comparison of these two fixation methods showed that Susa fixation gave larger diameters in the case of both the cell body and the nucleus, but the relations of these respective diameters to one another were the same after both Bouin and Susa fixation. The larger diameters here reported are, therefore, due to the fixation in Susa's fluid.

Tables 1 to 3 thus contain the data which have been used for determining the various relations of these three characters according to sex.

In order to show the relations according to sex of measurements of the cells, their nuclei, and the corresponding nerve fibers, four methods of comparison have been employed. The average results are recorded in table 4.

1. At like ages. No correction: A direct comparison of the observed areas in the males and in the females, at like

TABLE 3

Sectional areas of the largest fibers in the ventral root of 4" and 5" lumbar nerves—albino rat. K. Ide ('30), tables 5 and 5a

From table 5. 4" lumbar nerve				
	AGE. DAYS	BODY WT., GM.	BDY. LGTH., MM.	FIBERS, AREA SQ. μ
20♂	210	242	213	253
20♀	210	192	195	249
5" lumbar nerve				
20♂	210	242	213	249
20♀	210	192	195	249
From table 5a. 4" lumbar nerve. Sex excess computed				
5" lumbar nerve				
8♂	555	374	239	309
10♀	552	276	216	302

ages, has been made. In this instance the areas for the males in each group are the larger. The percentage values for the 'male excess' were determined for the three characters, cells, nuclei, and fibers, and these are entered in column 1 in table 4.

2. On body length in each sex: The observed areas of the cells was divided by the body length in millimeters. This gave an area of 1 mm. of length in each sex. The area thus found for the female was always larger than that for the male. The percentage value of this 'female excess' is given in column 2 of table 4.

3. On 'sectional body area': When the body weight is divided by the body length, the average weight, in grams, for 1 mm. of length is obtained. This weight may be transmuted into volume in cubic millimeters. When these are considered as forming a layer 1 mm. thick, we obtain a slice or section of the body having a given area. This is designated as the sectional body area. The observed cell areas in each sex were divided by the corresponding sectional body area, and

TABLE 4

Giving the percentage values of the 'excess,' according to sex, in the areas of the largest motor nerve cells and their nuclei, in the 4" and 5" lumbar segments of the spinal cord of the albino rat, and in the corresponding ventral root fibers. Based on the data for the cells, given in table 1; for the nuclei, given in table 2; and for the nerve fibers, given in table 3

Cells: areas. Percentage excess according to sex								
	1 AT LIKE AGES		2 ON BODY LENGTH		3 ON SECTIONAL BODY AREA		4 ON BODY WEIGHT VOLUMES	
	Male excess		Female excess		Female excess		Female excess	
Average	4.7		7.6		19.4		28.6	
Nuclei: areas. Percentage excess according to sex								
	Male excess		Female excess		Female excess		Female excess	
Average	0.85		11.7		23.6		37.6	
Fibers: areas. Percentage excess according to sex								
	Male excess		Female excess		Female excess			
	4" L	5" L	4" L	5" L	4" L	5" L		
Average	1.6	1.1	7.1	8.6	15.3	17.8		
Average 4"and 5"	1.3		7.8		16.6			

a value for 1 sq.mm. of 'sectional body area' obtained. The fraction for the female was always larger than that for the male, and the percentage values of the 'female excess' are given in column 3 of table 4.

4. On body weight: When the volume of the cells or of their nuclei is computed—the most direct comparison of these is with the corresponding body weights—considered as volumes in cubic centimeters. Dividing the observed volume of the cells in each sex by the corresponding body volume, a

fraction is obtained which is always larger for the female than for the male. The percentage values of the 'female excess' are given in column 4 of table 4.

No entry is made in column 4 in the case of the fibers, as this volume determination does not apply to them.

We turn to a consideration of the data in table 4, which deal only with the average excess according to sex for each of the characters considered.

In column 1—at like ages—the values are all somewhat higher for the male, i.e., there is a 'male excess.' The male is always the heavier and longer animal—and so shows larger nerve cells, nuclei, and fibers. When, however, at like ages, either the body length (column 2), the sectional body area (column 3) or the body weight (column 4) is used as a criterion, there is found a 'female excess.' Since the value of each of these three reference standards is greater for the male than for the female, and enough greater to more than compensate for the excess values for the cell areas or fiber size in the male of like age, the values for the female show an excess in all these instances.

In the case of the cell areas on body length (column 2) it appears (table 1) that the body length is about 12 per cent greater in the male. The average female excess for the three characters is always less than 12 per cent.

In the case of the cell areas on sectional body area (column 3), two factors are involved. The sectional body area is determined by body length, which we have just seen is 12 per cent greater in the male—and by body weight, which is 37 per cent greater in the male (table 1). The introduction of the body weight into the computation puts the male at a still greater disadvantage, and as a result the value for the 'female excess' (column 3) is more than twice that found for the comparison on body length alone.

In the case of the values on body weight (column 4), the difference in body weight (=volume) according to sex amounts, as just stated, to 37 per cent in favor of the male, and the 'female excess' shown for the volume of the cells and

of the nuclei is therefore the highest found. The three comparisons showing a female excess may be considered further.

The data based on body length can be neglected as partial.

The data based on sectional body area (a datum derived from body length and body weight) are better, but the data based on body weight (=body volume) in relation to cell volume give the most informing results, which can be considered trustworthy, since the percentage value of the weight of the musculature (43 to 44 per cent) is nearly the same for both sexes (Jackson and Lowry, '12; Donaldson, MS.), and thus the relations of the motor cell volumes are the same to the musculature as to the entire body. The volume relations of the motor cells to entire body give, according to our data, a female excess of 28 per cent for the cells and 37 per cent for their nuclei, and are the relations which we consider most significant.

However, the area determinations based on sectional body area are of value as showing how the sex differences in the areas of the cells and the nuclei stand to those for their respective fibers. It is seen at once in table 4 that the female excess for the area of fibers is similar to the female excess for the area of the cells, and it may therefore be inferred that in both sexes the same fraction of the cell surface is utilized for the formation of the axon. To be sure, the measurements of the fiber areas by Ide ('30) are based on the sections of the entire fibers, but in these mature animals half this entire area is represented by the area of the axon (Donaldson and Hoke, '05), so that the relations for the axons are identical with those for the entire fiber.

DISCUSSION

Observations on the size of the nerves, cells, and fibers in the rat, according to sex, have been reported by several investigators.

Dunn ('12) was apparently the first observer to call attention to the larger size of the nerve fibers in the female rat. She examined the fibers in the ventral root of the second

cervical nerve, and found them to be relatively larger in the female than in the male.

Ping ('21) found the largest cells and their nuclei, in the superior cervical sympathetic ganglion, larger in the female in both The Wistar Colony albinos and in Doctor King's inbred albinos. The same relations held good for the wild Norway rat (Ping, '21 a).

In his study of the inner ear of the albino rat, Wada ('23) found the cells and nuclei larger in the female in both the ganglion spirale and ganglion vestibulare.

Wang, in 1927, reported that the cross section of the optic nerve was larger in the female than in the male in both the albino and the gray Norway rat.

K. Ide ('29, '29 a, '30, '30 a) found the sectional areas of the median and sciatic nerves, and of the largest fibers in them—also the sectional areas of the lumbar nerve roots (ventral and dorsal) and the largest fibers in them—to be larger in the female than in the male albino rat.

H. Ide ('30, '30 a) determined the same relations for the median and sciatic nerves of man in both whites and negroes.

Ngowyang ('30) found, in the albino mouse, the diameters of the motor cells and their nuclei in the four divisions of the spinal cord to be greater in the female.

In 1931, Abe reported that when material from albino rats of like ages was used, the diameters of the Purkinje cells in the female were 97.5 per cent, and of the ganglion cells of the cerebral cortex, 94 per cent, of those for the corresponding male of like age. The like comparison of the diameters of the motor cells in our series show that, at like ages, the diameters in the female are 97.7 per cent of those for the male, thus showing approximately the same difference that Abe found for the Purkinje cells.

Reference to table 157, Donaldson ('24) shows that at like ages (365 days) the weight (= volume) relations between the body and the brain, when determined in the same manner as those in column 4, table 4, reveal a female excess for the volume of the brain by 16 per cent, and similarly for the cord

of 15 per cent. Thus, though the deviations according to sex are in the same direction for both these parts of the nervous system, they are, nevertheless, distinctly smaller in amount than those found for the motor cells as given in column 4, table 4. An interpretation of this difference must await the results of further study.

The foregoing citations indicate that in the female albino rat the sympathetic cells, the cells of some of the sensory ganglia, and the nerve fibers in the peripheral nerves, and in both the spinal nerve roots, are relatively larger in the female. In certain instances this same relation holds true for the gray Norway rat and for other species, as the mouse and man.

In the present instance a comparison of the area of the motor cells in the 4" and 5" lumbar segments was made on the basis of the sectional body area, as the measurements on the corresponding root fibers were available in these terms, and thus the size relation of one type of neuron—that representing the efferent system—could be determined.

CONCLUSIONS

As the result of comparing the sectional areas and volumes of the largest motor cells and their nuclei in the ventro-lateral column of the 4" and 5" lumbar segments of the spinal cord of the albino rat, it was found (table 4):

1. At like ages, the areas of the cells, nuclei, and fibers are somewhat greater in the male (column 1).
2. On 'body length' the areas of the cells, nuclei, and fibers are greater in the female (column 2).
3. On 'sectional body area' ($=$ body weight divided by body length) the areas of the cells, nuclei, and fibers are greater in the female (column 3). The percentage female excess is more than twice that given in column 2.
4. On body weight ($=$ volume) the volume of the cells and nuclei show the greatest female excess (column 4).
5. The comparison by volume is considered the most significant determination.

6. The comparison by sectional body area permits the introduction of the areas of the corresponding ventral root fibers, as determined by K. Ide ('30).

7. The increase in the area of the fibers according to sex is similar to that shown by the cells.

8. Combining the data for the cells and their nuclei with those for the fibers, a complete picture of the size relations of the motor neurons according to sex is obtained.

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STUDIES ON THE ANTERIOR HYPOPHYSIS

III. THE ANTERIOR HYPOPHYSIS IN VITAMINE E-DEFICIENT RATS

WARREN O. NELSON¹

*Washington Square College, New York University, and The Hull Zoölogical
Laboratory, The University of Chicago*

The effects of castration upon the cytological picture of the rat hypophysis has been described by Addison ('17). This author has shown that the basophiles enlarge markedly following gonadectomy, this enlargement finally attaining the so-called 'signet-ring' condition. Engle ('29) and Evans and Simpson ('29) consider this enlargement as indicating the storage of the gonad-stimulating hormone, because they were able to show that the hypophyses of gonadectomized rats are more potent than glands from normal animals in their capacity to stimulate the immature ovary. However, Emery's ('32) demonstration that the hormone occurs in the blood of castrated rats—a condition not present in normal animals—indicates that the gonad-stimulating factor is not confined to the hypophysis in castrated animals.

These studies, as well as a number of others, especially those of Moore and Price ('32), are evidence for the existence of a reciprocal relationship between the gonads and the anterior hypophysis. Thus the gonads are stimulated through the agency of the pituitary hormone, but the gonadal hormones exercise an inhibitory influence on the hypophysis. Removal of the pituitary causes gonadal regression, but gonadectomy permits abnormal pituitary activity.

Inasmuch as gonadectomy in the male removes all of the testicular elements, it becomes of interest to inquire into the gonad-pituitary relations of animals in which only certain

¹ National Research Council Fellow

components are eliminated. Such a condition exists in cryptorchid, x-irradiated, and vitamine E-deficient male rats where the cells of the germinal line are abolished. The various researches of Mattill ('24), Mason ('26), and Evans and Burr ('27) have established the pathology of E-deficiency. It has been shown that male rats become completely sterile after being maintained for some time on an E-deficient diet. The testes assume an appearance similar to that described by Moore ('24) as occurring in experimental cryptorchid testes. The degeneration of the germ cells progresses in the inverse order of their formation. Mason has described five stages in the degeneration of the germinal epithelium in E-deficient males. He has been able to show that, although at least 3 months on the basal ration employed in his studies are necessary for complete degeneration, less than 50 days are required for the completion of the regressive changes after the process has been initiated. The interstitial cells apparently are not affected by the absence of vitamine E.

The profound effects of E-deprivation on the male gonads makes it of some interest to inquire into the condition of the hypophysis in vitamine deficient animals. There is reason to suspect that the testicular condition established by E-deficiency in the male might be reflected in the histological picture of the anterior hypophysis. In the female, where the reproductive failure concerns only the inability to maintain full-term gestations, the hypophysis should be normal. In connection with the histological study it was considered of interest to examine the effects of fresh anterior lobe implants upon the ovaries of immature rats. Any hypophyseal change, such as occurs following castration, should be apparent in the capacity to stimulate the immature ovary.

The diet employed in this study consisted of casein 18 parts, cornstarch 54 parts, lard 22 parts, cod-liver oil 2 parts, and salts 4 parts. In addition, each rat received 0.4 to 0.6 gm. of yeast daily. This ration is identical with Evans and Burr's basal ration 51 and was also used by Kudrjashov ('31) in his vitamine E studies.

All of the male animals and a majority of the females used in this study comprised litters which had been born to females and sired by males on the basal ration before sterility had supervened. Animals of both sexes were sacrificed at intervals throughout the course of the experiment. The hypophyses, gonads, and in some instances the sex-accessory organs were removed and fixed in Zenker-acetic or Zenker-formol-osmic. Krichesky's ('31) modification of Mallory's triple stain was used for most of the staining, although Harris' hemotoxylin and erythrosin were employed in some instances.

HYPOPHYSES FROM E-DEFICIENT MALES

Twenty-six males reared on the basal ration from the day of weaning were employed in this phase of the study. The pituitaries of males sacrificed during the first 4 months of life on the basal ration showed no appreciable or constant deviations from the normal histological picture. However, during the fifth month alterations in the direction of the castrate type became apparent. This condition was accentuated during the ensuing 4 months, so that in males maintained on the basal ration for 9 months the histological picture in the hypophysis showed a regular and definite increase in the size and number of basophiles. This alteration did not approach the 'signet-ring' condition² nor was there any apparent decrease in the number of eosinophiles such as Addison has described in long-time castrates.

The degenerative changes occurring in the testes of these males correspond in general with the descriptions given by

² It is well, perhaps, to emphasize this difference in view of the extremely loose manner in which the term 'castration cell' has been employed. Since castration, in the rat, affects the basophilic cells principally, it is apparent that these modified basophiles assume numerous intermediate and closely related conditions before the 'signet-ring' stage is attained. In early castration the cells undergo a progressive enlargement and an increase in number. Although these changes and the subsequent vacuolization are gradual, all of these cell forms, at one time or another, have been termed 'castration cells.' The writer believes that it is preferable to reserve that term for the vacuolated cells which are present in the more advanced stages of castration.

Mason, Evans, and Burr, and Kudrjashov. These changes have been adequately considered by the above authors and need not be repeated here. Their reports show differences in the rate of testicular degeneration and the attainment of Mason's stage 5. These differences are probably due to minute variations in the amount of vitamine E present in the basal rations and to variations in susceptibility to E-deprivation on the part of different animals.

In the present study the testes of three males fed the basal ration for 90 days after weaning presented some variation. In one case there was no tubular degeneration beyond stage 1, while in the other two animals practically all tubules were in stages 3 and 4. All four animals sacrificed at 120 days showed testicular degeneration to stages 4 and 5. Animals maintained on the basal ration for 150 days and longer showed complete degeneration of the germinal epithelium in by far the majority of tubules.

HYPOPHYSES FROM MALES RETURNED TO NORMAL FOODS

Two males were returned to the regular colony ration after having been maintained on the basal ration for 60 days. These animals were sacrificed 120 days later. Their testes and hypophyses were essentially normal. One male was returned to the ordinary foods after 100 days and a second animal after 120 days on the basal ration. They were sacrificed 110 days later. In each instance the testes were in stage 5 and the hypophyses showed definite changes toward the castrate pattern.

HYPOPHYSES FROM E-DEFICIENT NON-PREGNANT FEMALES

A study of the hypophyses obtained from nine non-pregnant females sacrificed after 123, 164, 203, and 235 days of life on the basal ration demonstrated an entirely normal picture. Each of these females had been shown definitely to be deficient in vitamine E by the occurrence of at least one resorption gestation. The oestrous cycles of these females were normal in character and their ovaries, uteri, and vaginas were char-

acteristic of the particular phases of the cycle in which the animals were sacrificed.

HYPOPHYSES FROM E-DEFICIENT PREGNANT FEMALES

Hypophyses from twelve E-deficient females sacrificed at intervals between the fifth and the twentieth day following coitus were examined. Each of these females had experienced at least one failed gestation prior to the one during which they were sacrificed. The hypophyseal picture seen in these females appeared to be identical with that observed in normal pregnancies. Furthermore, the administration of progesterin to a series of pregnant E-deficient females, in an effort to prevent foetal death (Nelson, '31) did not modify this picture.

HYPOPHYSES FROM GONADECTOMIZED E-DEFICIENT RATS

Two males and two females were gonadectomized 3 months after they had been placed on the basal ration. They were sacrificed 5 months later. The hypophyses from both sexes showed the typical picture seen in ordinary 5-month castrates. It should be emphasized that in the male this condition is much more pronounced than in non-castrates maintained on the basal ration for an equivalent period. In the female, of course, an entirely new hypophyseal picture is produced.

IMPLANTATION OF ANTERIOR HYPOPHYSES FROM VITAMINE E-DEFICIENT RATS IN IMMATURE FEMALE RATS

Five immature female rats 24 days of age were each given one hypophysis daily for 3 days. They were sacrificed on the fifth day following the initial implantation. All implants were made intra-muscularly in the manner described by Smith and Engle ('27). The animals employed as donors were males and females which had been maintained on the basal ration for approximately 230 days. Parallel with these implantations, five immature females were given pituitaries from rats fed on the regular colony ration. These donors were selected to correspond as closely as possible with the

E-deficient donors in age and weight. In the following table the immature rats receiving implants from E-deficient males, from normal males, and the untreated controls were litter-mates. The animals receiving implants from E-deficient females, from normal females, and their controls were from a second litter. Data from animals of a third litter are given also in the table. Two of these animals received implants

TABLE 1

ANIMAL NO.	AGE	WEIGHT	PITUITARY IMPLANTS		AUTOPSY DATA			
					Age	Body weight	Weight ovaries	Weight uterus
	Days	Grams	Donor	Number	Days	Gm.	Mg.	Mg.
E ₁	24	39	E-deficient males	3	28	46	91.4	103.
E ₂	24	40	E-deficient males	3	28	49	98.7	121.6
E ₃	24	38	E-deficient males	3	28	48	103.6	93.4
N ₁	24	40	Normal males	3	28	50	61.7	129.1
N ₂	24	41	Normal males	3	28	49	48.2	156.7
N ₃	24	39	Normal males	3	28	47	54.7	138.9
		42	Control—					
C ₁	24		no implants	0	28	52	18.3	26.3
		40	Control—					
C ₂	24		no implants	0	28	49	14.9	24.2
E ₄	24	42	E-deficient females	3	28	53	26.3	89.7
E ₅	24	43	E-deficient females	3	28	52	28.6	92.3
N ₄	24	42	Normal females	3	28	50	24.5	80.6
N ₅	24	44	Normal females	3	28	55	30.8	115.2
		45	Control—					
C ₃	24		no implants	0	28	56	19.6	26.2
		43	Control—					
C ₄	24		no implants	0	28	52	15.4	29.5
G ₁	24	44	Castrated males	3	28	56	144.2	118.4
G ₂	24	42	Castrated males	3	28	51	158.5	129.8
N ₆	24	45	Normal males	3	28	57	64.6	143.2
		45	Control—					
C ₅	24		no implants	0	28	54	14.8	23.5

from male rats which had been castrated 4 months earlier, one received implants from normal males, and the fourth was an untreated control.

From the tabulated data it is apparent that anterior pituitary implants from vitamine E-deficient male rat donors are more potent for the gonad-stimulating hormone than are glands from normal males, but they are not as rich in the

hormone as glands from males castrated for 4 months. On the other hand, anterior hypophyses from E-deficient female rats and those from normal females are about equal in their capacity to stimulate the immature ovary.

DISCUSSION

The effects of E-deprivation upon the germinal epithelium of the testes and their reflection upon the histological picture of the anterior hypophysis are in accord with other reports relative to testicular damage. Mottram and Cramer ('23) studied the testes and hypophyses of rats which had been exposed to radium treatment. They found that the germinal epithelium underwent intense atrophy and that the anterior hypophyses exhibited typical castration changes. Furthermore, they advance the idea that the testis produces two hormones, one elaborated in the interstitial cells and the second produced in the germinal epithelium. Martins and Rocha ('31) found that in experimental cryptorchid male rats, where the germinal epithelium is degenerated, castration changes are present in the hypophysis. They, too, believe that the testis produces a dual secretion and have named the hormone of the germinal epithelium 'Andrina.'

The writer has examined the hypophyses from a series of male rats that had been in an experimental cryptorchid condition for periods of time ranging from 3 weeks to 5 months. The histological picture in hypophyses from 5-month cryptorchids is quite similar to that observed in E-deficient males. In neither condition are the histological changes as pronounced as they are in males castrated for 30 days. Martins and Rocha designate the condition in cryptorchid animals as an 'attenuated castration.' In the vitamine-E and cryptorchid material studied by the writer it can be said that no castration cells were present if we define a castration cell, in the rat, as one which exhibits a definite vacuolization and development toward the 'signet-ring' condition (vacuolization ordinarily occurs during the second month of castration). It would be more nearly correct to say that the hypophyses of

the E-deficient and experimental cryptorchid males reported here showed 'castration changes,' since the earlier effects of castration on the hypophysis, viz., hyperaemia, and increase in size and number of the basophilic cells, were present.

Van Wagenen ('25), in a brief report, stated that the pituitaries of male rats deprived of vitamine X (now called vitamine E) show castration cells. She also reported the occurrence of a castration hypophysis in males subjected to ligation of the vasa efferentia.

All of the above conditions produce similar testicular lesions, viz., degeneration of the germinal epithelium, and through this testicular damage induce the occurrence of castration changes in the hypophysis. When it is remembered that the sex accessories remain normal in these animals, it would appear that the original suggestion of Mottram and Cramer is of some significance. If, as they contend, the germinal epithelium elaborates a hormone which exercises a control over the anterior hypophysis, it becomes apparent that the gonad-pituitary relationship in the male is not as simple as might be supposed. On the other hand, if the existence of a second testicular hormone is assumed, a number of otherwise puzzling problems may be explained. For example, the appearance of castration changes in the pituitaries of animals which have normal sex accessories is reconcilable. Other instances are: 1) The demonstration by Steinach ('16) that ovaries grafted in male animals do not function, and the reports of Sand ('18) and Smelser ('33) that either castration or abdominal replacement of the testes will permit functional activity of the graft. 2) Martins and Rocha's demonstration that experimental and cryptorchid males exert the same effect upon the oestrous cycles of parabiotic female partners. A related experiment by Witschi, Levine, and Hill ('32) wherein x-irradiated males were placed in parabiosis with normal females. 3) The demonstration that hypophysis from cryptorchid (Evans and Simpson, '29) and E-deficient males are more potent in gonad-stimulating hormone than those from normal animals. All of these experiments can be

explained and correlated if the existence of a testicular hormone produced by, or at least with the aid of, the germinal epithelium is assumed. The evidence at hand indicates that it is concerned only with the anterior hypophysis.

While it is apparent that all of the above observations may be explained on the basis of a special hormone secreted by the germinal epithelium, a second and not unlikely explanation may be advanced. It is quite possible that the normal quantitative production of the testicular hormone, presumably by the interstitial cells, is not carried on in the absence of the germinal epithelium. Thus the altered production of the testicular hormone may be sufficient to maintain the accessory organs in a normal or approximately normal condition, but not be sufficient to maintain the anterior hypophysis in its normal histological and physiological state. Some experiments of the writer (Nelson, '33) concerning the effect of graduated doses of testicular hormone upon the hypophyses of castrated rats have resulted in the production of a gradation in the resulting histological pictures. It has been shown that a dosage which will maintain the normal cytological condition of the seminal vesicles and prostate will not prevent entirely the occurrence of castration changes in the anterior hypophysis. If the dosage was increased beyond that necessary for accessory maintenance, these castration changes were prevented. These experiments may be considered as supporting the conception of a lowered testicular hormone production in animals deprived of their germinal epithelia, but their principal value in the present connection lies in the writer's desire to emphasize the necessity for caution in unreservedly accepting the contention that the testes produce two separate and distinct hormones. The answer to that question must await further biochemical and biological investigation.

The employment of hypophyses from E-deficient rats as fresh implant material furnishes further evidence that the profound effects of E-deprivation upon the testis are reflected in the anterior pituitaries of male animals. Glands from

E-deficient male animals are more potent than glands from normal males, but less so than pituitaries from males castrated for 4 months. Engle ('29) has reported that castration in male and female rats results in a greatly increased capacity on the part of their hypophyses to stimulate the immature ovary. Evans and Simpson ('29) confirmed this report and showed that the hypophyses from experimental cryptorchid male rats were more potent than normal glands, but less potent than glands from castrated males. This report is of especial interest in the present connection since the same relation was shown to be true for the hypophyses of E-deficient males. The similarity in the gonad-stimulating capacity of cryptorchid and E-deficient male rat pituitaries is not surprising, in view of the similar testicular condition of the two classes.

A few words should be said concerning the condition of the male sex accessories (seminal vesicles and prostate) in E-deficient animals. It is well known that these organs depend upon the presence of the testes for the maintenance of their normal function, and it has been generally assumed that the hormone responsible for their maintenance is a product of the interstitial cells. Experimental cryptorchid males have normal accessories and the same is true of males sterilized by x-irradiation. Evans and Burr ('27) state that the accessories are normal in males which have been rendered completely sterile by E-deprivation. However, they do note that in males maintained on the basal ration for 13 to 14 months the power to form the vaginal plug is lost. This condition is indicative of some disturbance in the accessory organs. Mason (personal communication) states that, although in a few of his animals the accessories showed some indication of atrophy, the majority possessed normal seminal vesicles and prostates when autopsied. He suggests that the occasional occurrence of atrophy may be due to inanition effects and not to the lack of vitamine E. The inanition and vitamine-B studies of Moore and Samuels ('31) are in support of this possibility. On the other hand, Kudrjashov ('31), who made

a study of the accessories in males on an E-deficient diet, claims that they show degenerative changes after 145 days on the basal ration and a castration condition after 200 to 240 days. On the basis of this work, he believes that the interstitial cells do not produce the testicular hormone, at least in the absence of the normal cell elements of the seminiferous tubules.

In the present study a detailed examination of the accessories was not undertaken, but the accessories which were examined confirm the observations of Evans and Bishop and Mason that in males whose testes exhibit a widespread degeneration of the germinal epithelium the sex accessories are normal. However, apparently no one has made a cytological study of the accessories using the precise methods for the rat developed by Moore, Price, and Gallagher ('30), and Moore, Hughes, and Gallagher ('30). Until this is undertaken, a definite statement concerning the condition of the accessories in E-deficiency cannot be made. The evidence at hand indicates that the seminal vesicles and prostate may retain a structural and functional normality in males whose testes have undergone irreparable tubular damage, a damage that is reflected in the anterior hypophysis by the occurrence of castration changes.

SUMMARY

The anterior hypophyses of various classes of rats maintained on a diet deficient in vitamine E were examined histologically and physiologically.

It was shown that definite castration changes occur in the hypophyses of males, but that these changes do not attain the condition seen in the pituitaries of castrated animals. The hypophyses of non-pregnant and pregnant females were shown to be normal for the particular condition of the animal at the time of sacrifice.

The employment of hypophyses from E-deficient animals as fresh implants demonstrated that male glands are more potent for the gonad-stimulating hormone than pituitaries from normal animals, but less potent than glands from cas-

trates. Female pituitaries were quite comparable to glands obtained from normal females.

The question of a dual secretion on the part of the testis is discussed, together with various studies which have indicated the occurrence of a hormonal imbalance following the destruction of the germinal epithelium.

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INTRANUCLEAR CRYSTALLOIDS IN THE SUPERFICIAL EPIDERMAL CELLS OF THE CATFISH, *AMEIURUS NEBULOSUS*

ALDEN B. DAWSON

Zoölogical Laboratories, Harvard University

ONE PLATE (TEN FIGURES)

Although the nucleus has long been recognized as a major center of cellular activity, it rarely contains formed inclusions other than nucleoli. For this reason, the presence of other structures within it attracts immediate attention.

Crystal-like bodies are found within the nuclei of the superficial cells of the epidermis of the catfish (fig. 10) and are uniformly present in all cells bearing a striated border. Occasional cells which appear to lie deeper in the epidermis may contain similar structures, but these cells probably are actually superficial, the lack of any readily observable connection with the surface being due to the plane of the section. Furthermore, the distribution of intranuclear crystalloids appears to be limited to the superficial ectodermal cells. They are not present, however, in the superficial cells of the epithelium of the lateral-line canal and lateral-line pores, although they are found in the epithelium of the roof and floor of the mouth.

The crystalloids were first observed in fresh scrapings from the skin of the catfish, and appeared as clear, highly refractile bodies. When such scrapings were dried on the slide and subsequently stained by Wright's method for blood films, the crystalloids remained uncolored (figs. 1 to 9). In sections, following fixation in Helly's fluid, they were stained densely with both Mallory's phosphotungstic acid hematoxylin and Heidenhain's iron hematoxylin.

The crystalloids (figs. 1 to 9) are relatively constant in length, although there is some variation in this respect. In general, they appear lath-shaped, but their small size hinders the determination of their exact shape. They vary in width, a few being narrow and almost needle-like. Some of these differences in width may be due to their being viewed either on edge or from varying angles. The crystalloids are usually straight, but occasional sinuous forms were noted (fig. 8).

The number found in any one nucleus is variable. They sometimes occur singly, but as many as 10 may be present. They may lie parallel or cross each other at varying angles. They do not appear to deform the nuclei to any great extent.

The chromatin content of the nuclei containing these special inclusions does not differ appreciably from that of the nuclei of deeper lying cells, and a single, small, spherical nucleolus appears to be characteristic of all epidermal cells.

No attempt has been made to determine the physical and chemical make-up of these bodies. Their small size makes optical studies difficult. Moreover, it is doubtful if they play any important role in nuclear metabolism. Their restricted distribution, in the superficial cells, suggests that their presence in the nuclei may be associated with senile or degenerative changes within the cells.

Intranuclear crystalloids are encountered rather frequently in plants, as shown by the early studies of Zimmerman (1896), but have rarely been reported in animals. Grandis (1889) described crystals found in the nuclei of the liver and kidney of the dog and these have been the subject of much study and speculation ever since (Browicz, 1897; Herring, '06; Brandts, '09). These crystals were long suspected of having been derived from hemoglobin, but the most recent paper of Berg ('29) contains the suggestion that they may be formed from allantoin.

Other crystals have been described for the sympathetic and spinal ganglion cells of mammals, especially the hedgehog (Sjövall, '01), but no satisfactory explanation of their source has been given. Crystals within the nuclei of the intestinal

epithelium of the larvae of *Tenebrio molitor* were described by Magshikowskaja ('29), who regarded them as a food reserve, since they could be depleted by starvation. On the basis of their solubilities and digestibilities, he thought the crystals were probably protein. Crystal-like bodies of nucleolar origin are also present in the yolk cells of the turbellarian *Prorhynchus applanatus*, and are believed to constitute a source of yolk (Jones, '31).

A study of available literature indicates that the occurrence of intranuclear crystalloids in animals is not characteristic of any particular tissue, being reported in nerve cells, intestinal epithelium, hepatic and renal cells, and epidermal cells. Furthermore, they are not characteristic of groups of animals, but occur sporadically in widely separated classes and phyla. No satisfactory explanation of these intranuclear bodies has been given. They are apparently the result of some peculiar metabolic condition existing in certain tissues of a very limited number of animals.

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PLATE 1

EXPLANATION OF FIGURES

1 to 9 Isolated epidermal cells obtained by gently scraping the surface of the skin; fixed by drying and stained with Wright's method for blood. They are selected to show the variations in size, form, number, and distribution of the intranuclear crystalloids.

10 Superficial portion of the epidermis seen in vertical sections, showing the appearance and distribution of the crystalloids. Helly fixation; Mallory's phosphotungstic acid hematoxylin.



THE STRUCTURE OF THE SINUSES IN THE LYMPH NODES

CECIL K. DRINKER, GEORGE B. WISLOCKI, AND
MADELEINE E. FIELD

*Department of Physiology, Harvard School of Public Health, and Department
of Anatomy, Harvard Medical School*

THREE PLATES (TEN FIGURES)

In the course of a series of experiments on the filtering capacity of lymph nodes for bacteria and for foreign particles of various sizes, it was found necessary to get more exact information on the character of the sinuses than was available in the literature. Hellman ('30) has summarized the divergent opinion in regard to lymph-sinus structure. It is, first, a matter of controversy as to whether the sinuses possess an endothelial lining of the same type as lymphatics in other regions, or whether the wall is formed by reticulum cells which in this position become plate-like. Added to this disagreement, we have a further source of confusion in the discussion as to whether the walls of the sinuses—however formed—are complete, or whether they display openings leading into the lymphoid tissue.

During the past year, in order to be sure that our experiments on filtration by the popliteal and iliac lymph nodes were carried out under conditions of lymph pressure and flow normal to the dog, we have cannulated an afferent vessel at the popliteal node, and measured the pressure under normal conditions of activity. The dogs employed were anesthetized with nembutal (sodium-ethyl (1-methyl-butyl) barbiturate), and heparinized so that the lymph flow became incoagulable. A large afferent lymphatic in the popliteal space was then exposed and cut in two places, so as to permit the introduction

of a T-cannula. When tied in place, the lymph flowed through the top of the T, and the upright arm was connected to a mercury manometer. With the leg of the dog at rest, no pressure developed. When the foot was attached to a rotator, so as to provide passive motion, pressures from 10 to 25 mm. Hg were registered.

In the injections described in this paper, adult dogs—in no case old animals—anesthetized with nembutal have been injected with suspensions of Higgins' India ink diluted with salt solution, and with an acacia-graphite mass of appropriate dilution. The injections have been run in through quartz cannulas tied in lymphatics along the saphena parva vein just above the ankle. These lymphatics enter the popliteal node, and eventually the fluid they carry traverses the iliac nodes on the same side. Injections were made with a syringe attached to a manometer, and pressures were kept within normal range. As a consequence, the deposition of injected material can be relied upon to fall in positions normal for lymph traveling through the gland. Injections required not longer than 10 minutes. The animal was then bled to death and the popliteal and iliac nodes fixed in neutral formalin, Zenker's solution, or Bouin's fluid.

Should it be thought that non-dialyzed Higgins' India ink may of itself have been toxic, it may be pointed out that exactly similar results were obtained with the acacia-graphite mixture, which consisted of nothing save clean graphite particles suspended in an acacia-Ringer solution.

RESULTS AND DISCUSSION

Figures 1 and 2 are low-power photomicrographs of injected popliteal nodes. They display the general distribution of the ink mass, and indicate the fact that distribution is not always uniform. It is also clear that the lymphoid tissue is easily penetrated by the injected ink. Figures 3, 5, and 8 show still more clearly that the lymphocytic collections, especially in the cortex of the node, are easily penetrated by fluid in the marginal sinus.

When the marginal sinuses are studied closely, one finds occasional regions, such as are seen in figure 6, where the sinus wall, upon the inner nodal side, is fairly regular, though on high-power examination only a poorly defined lining membrane can be detected. The more usual situation in the marginal sinus is shown in figure 8. Here one sees the strands of reticulo-endothelial tissue which cross the sinus and give a latticed appearance to it. They are heavily coated by the ink injection. Inside this latticed zone, and extending a considerable distance into the lymphoid tissue, there is a deposit of ink which has entirely escaped the sinus in spite of a vague sort of margin composed of a condensation of reticulum fibers which may be observed at certain points.

In the intermediary sinuses, particularly in the center of the node, or it would perhaps be better to say in regions where collections of lymphocytes are not large and the individual cells not closely packed, the injection mass runs between quite definite borders. This fact is shown in figures 4 and 7. These illustrations are of sinuses in the central portion of such a node as is shown in figure 1.

In figure 9 we have a higher magnification of an intermediary sinus in the center of a node injected with a very dilute solution of ink. In this particular sinus the endothelial cells have also phagocytized red blood cells. It is apparent that the wall of the sinus consists of a very definite layer of flattened cells resting upon a delicate lamina of reticulum fibers. The major part of the sinus lining resembles capillary or lymphatic endothelium. But at many points in the wall, two of which are shown under higher magnification in figure 10, the lining cells have phagocytized red cells. Kiyono ('14) showed that the lining endothelial elements of the sinuses in the lymph nodes of rabbits became filled with lithium carmine in vitally stained animals. This reaction is not characteristic of the endothelial cells of normal adult blood capillaries nor of the endothelium of lymphatics outside lymph nodes, unless the lymphatics are in rapid growth. Evans ('15) has mentioned the tendency of vascular endothelium to become phagocytic

in inflammatory areas, and Wislocki ('16) has shown that the lymphatic endothelium in rapidly growing tadpoles will stain vitally with trypan blue. Phagocytosis and vital staining are not characteristic of normal adult vascular or lymphatic endothelium, and the demonstration of Kiyono, to which our confirmatory observations may be added, should suffice to show that the endothelium lining the sinuses of the lymph nodes is not similar to that lining the vessels entering and leaving the nodes. It may be suggested from observation of figure 10 that a situation similar to that in the sinusoids of the liver is found in the sinuses of the lymph nodes, and that the occasional phagocytic cells in the lining membrane are analogous to Kupffer cells in the liver. The cells lining the sinuses of the lymph nodes, as well as those which form a lattice in the lumen of the sinuses, differ somewhat from the endothelial cells and the associated stellate cells of the liver sinusoids, in that the phagocytic cells of the lymph-sinus are continuous with a fine but extensive network of reticulum fibers and fibrils. The presence of attendant reticulum fibers does not necessarily exclude these cells from an endothelial origin, since the work of Kon ('08) and Corner ('20) indicates the ability of endothelial cells to produce reticulum fibers. In the case of the liver, however, the reticulum fibers are not numerous and are laid down solely between the endothelium and the subjacent liver parenchyma.

Maximow ('30) states that the sinuses in the lymph nodes are incompletely closed, and maintains that the lining cells as well as those contained in the sinuses are true reticulum cells and not endothelial in origin. We believe that the present physiologically controlled injections afford more exact evidence of the nature and degree of patency of the walls of the sinuses than has been heretofore brought forward. In this regard, Maximow says that the flattened cells, simulating endothelium, are more conspicuous in those localities where the proliferation of lymphocytes encroaches upon and compresses the sinus wall. In our experience, the opposite is true; where the lymphocytes are prolific in their growth as

in the cortex of the nodes, the walls are frequently incomplete, while in the medullary cords, which are by contrast relatively acellular, the contour of the sinus is outlined by a complete lining of flattened cells.

The present method of injection brings out one other interesting point in regard to the nature of the lymph-sinus. It may be seen by consulting figures 9 and 10 that the foreign particles upon reaching the sinuses tend without fail to become adherent almost immediately to the reticulum fibers and cell-processes of the reticulo-endothelial meshwork. This quality of the cells whereby adhesion of foreign particles takes place almost instantaneously must facilitate prompt and complete phagocytosis of foreign material. It is of interest that the particles are not first agglutinated into clumps, but seem to attach themselves to the reticulo-endothelial cells as separate, discrete particles. In certain blood vascular beds, on the contrary, according to the observations of Mrs. Lewis ('25), carbon particles become definitely entangled and clumped in a plasma clot before they are taken up by the phagocytic cells.

SUMMARY

1. Lymph nodes of young adult dogs have been injected with India ink and acacia-graphite masses under conditions of pressure and flow normal for the animal.

2. The walls of the sinuses in the injected nodes are formed by reticulo-endothelial cells, not by lining endothelium similar to that in the afferent and efferent vessels of the nodes.

3. The sinus walls are incomplete wherever lymphocytic growth is active, and lymph passing along the sinuses runs for varying distances into the lymphocytic collections.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

- 1 and 2 Low-power photomicrographs of injected popliteal nodes. $\times 6$.
- 3 The distribution of the injection mass in the cortical and in the beginnings of the intermediary sinuses. $\times 26$.
- 4 Intermediary sinuses in the medulla of an injected node. $\times 128$.

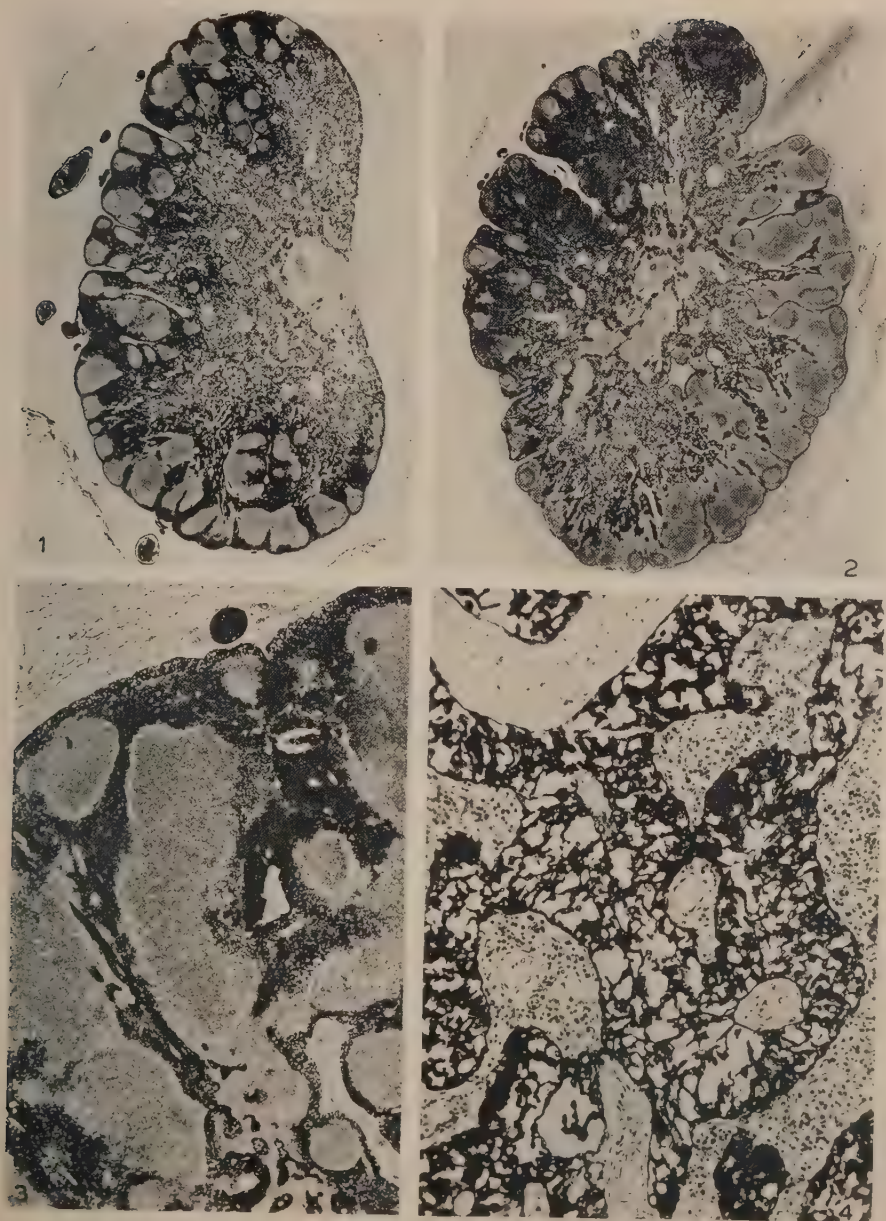


PLATE 2

EXPLANATION OF FIGURES

5, 6, and 8 The distribution of the injection mass in the cortical sinus. Notice the variation in the sharp margination of the sinus. $\times 70$.

7 Intermediary sinuses in the medulla of a gland, showing sharp margination. $\times 61$.

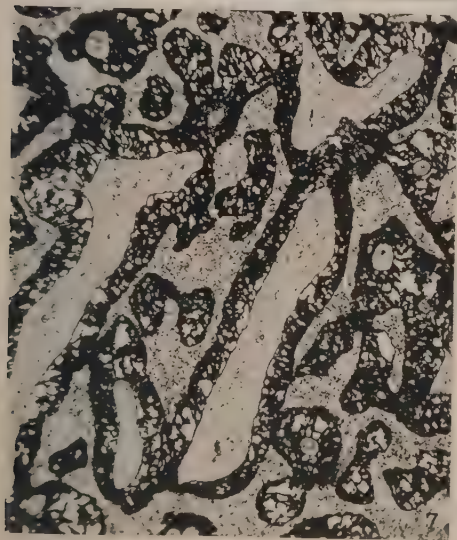
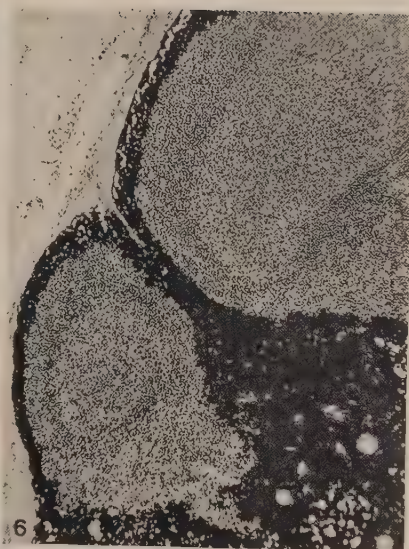
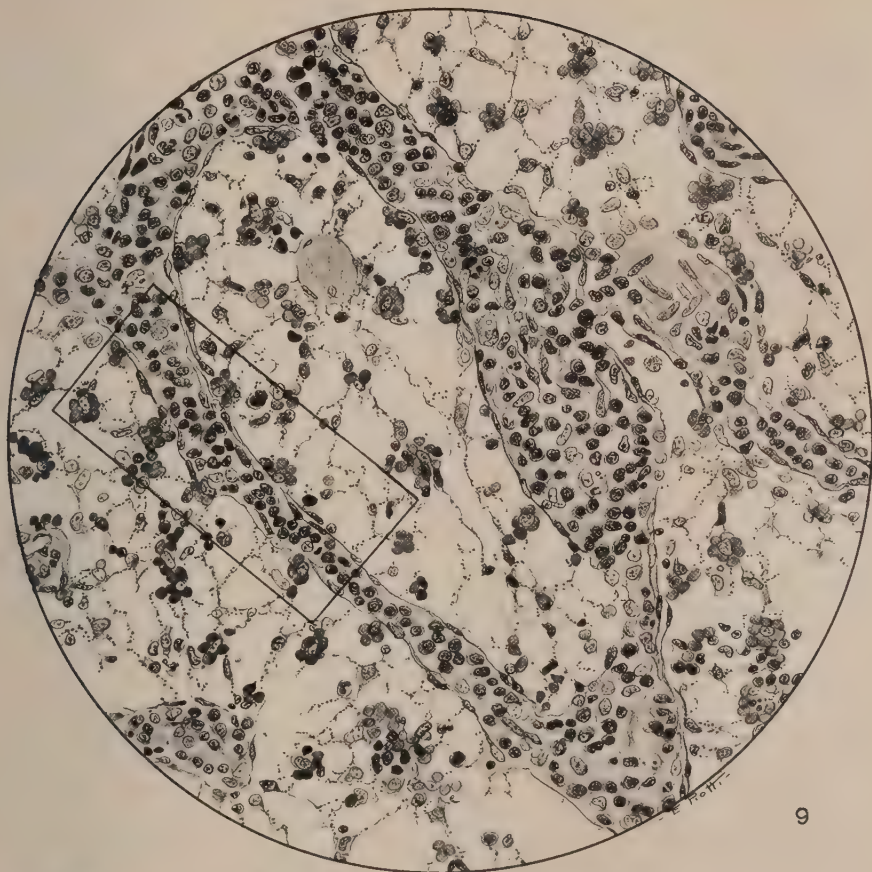


PLATE 3

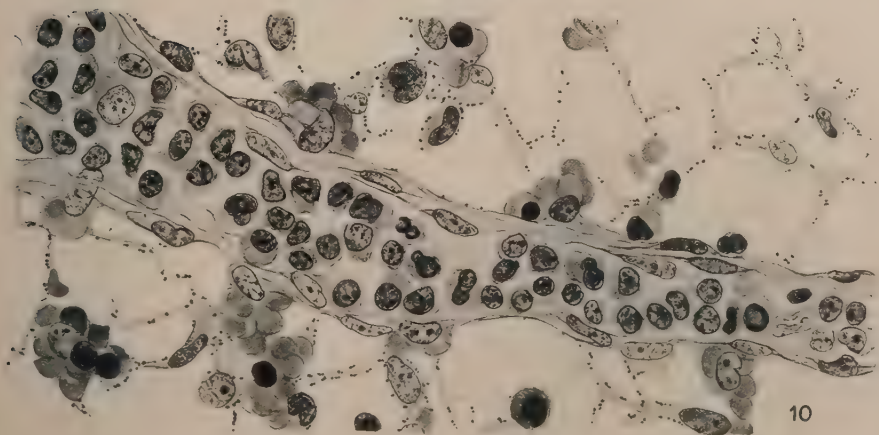
EXPLANATION OF FIGURES

9 Camera lucida drawing of an intermediate sinus injected with a very dilute India ink solution. Note the phagocytosis by the reticulo-endothelial cells in the sinus and in the wall, and also the flattened cells which line the sinus with fair completeness. $\times 390$.

10 Camera lucida drawing of the rectangle marked in figure 9. Details of reticulo-endothelial cells lining the sinus. $\times 1000$.



9



10

THE DEVELOPMENT AND HORMONAL CONTENT OF FETAL HORSE GONADS

H. H. COLE AND G. H. HART

College of Agriculture, University of California, Davis

AND

W. R. LYONS AND H. R. CATCHPOLE¹

Department of Anatomy, University of California, Berkeley

ONE TEXT FIGURE AND TWO PLATES (FOURTEEN FIGURES)

The development of fetal horse gonads assumes a new significance in view of the singular hormonal concentrations in the blood serum and urine of the pregnant mare. Fetal horse gonads have been studied by a number of investigators and it is strange that the striking findings of the earlier workers have not been more frequently cited. Before taking up a review of this work, however, we will state briefly the facts known regarding the hormonal concentration in the mare.

There is a very high concentration of the gonad-stimulating hormone in the blood serum of the mare between the forty-fifth and eightieth days of pregnancy (Cole and Hart, '30). The hormone may be recognized at about the fortieth day (there is a difference of about 5 days in the time at which it first appears in different individuals ranging from the thirty-seventh to the forty-second day of pregnancy). It disappears from the blood serum at about the one hundred and fiftieth day of pregnancy (one hundred and thirtieth to one hundred and eightieth). The time at which the concentration of this hormone becomes greatest varies considerably in different individuals—probably as much as 30 days. The time of maxi-

¹ Research scholar. Ministry of Agriculture and Fisheries of Great Britain.

imum concentration is reached, therefore, between the fiftieth and eightieth days of pregnancy. The range in time at which the hormone appears in different individuals is small, but the range in the time of the greatest concentration and also in the time of its disappearance is rather large. The hormone is present in the blood stream in recognizable amounts for about one-third of the gestation period. It is only rarely found in the urine. On the other hand, in the pregnant woman the concentration is greater in the urine than in the blood and, furthermore, the hormone is present during the greater part of pregnancy.

As a consequence of this high concentration of the gonad-stimulating hormone in the serum of the mare, there is a great follicular development of the ovaries, followed either by ovulation and the formation of true corpora lutea or by the formation of corpora lutea atretica, the latter undoubtedly predominating. This great ovarian development is followed by regressive changes so that in the last third or fourth of pregnancy the mare ovaries are devoid of large follicles and corpora lutea (Cole, Howell, and Hart, '31). In other words, with the exception of a few small follicles, the ovary would appear to be a quiescent organ.

Oestrin never appears in large amounts in the blood serum of the mare (Cole and Hart, '30 a). In late stages of pregnancy, 25 or 30 cc. of serum must be administered to a spayed rat to elicit a response. Large amounts of citrated blood must also be used. On the other hand, oestrin occurs at this time in abundant amounts in the urine of mares, as demonstrated by Zondek ('30). However, a careful study of the concentration of oestrin in the urine throughout pregnancy has not been made. Inasmuch as the ovary appears inactive in late stages of pregnancy, the source of this large amount of oestrin in the urine is an interesting study.

The known facts regarding the concentration of the sex hormones and the ovarian changes during pregnancy in the mare may be summarized as follows:

Gonad-stimulating hormone:

Appears in blood serum between the thirty-seventh and forty-second day.

Highest concentration in blood serum reached between the fiftieth and eightieth days.

Disappears from blood serum between the one hundred and thirtieth and one hundred and eightieth days.

Appears occasionally in small amounts in the urine.

Oestrin:

Concentration always small in blood.

Concentration high in urine. First appears between the sixtieth and one hundred and seventy-fifth days of pregnancy.

Ovarian changes:

Great development of ovaries beginning at about the forty-fifth day. Formation of ripe follicles either results in ovulation and the formation of corpora lutea or in the formation of corpora lutea atretica.

Regression of corpora after gonad-stimulating hormone disappears from blood stream so that by the last third or fourth of pregnancy the ovaries contain only small follicles.

LITERATURE ON FETAL HORSE GONADS

Franz Leydig (1850) described the histology of the testis in the newborn foal, and was especially interested in the masses of pigment-containing interstitial cells which he had also studied in other mammals. He noted their relation to the vascular channels which, as other investigators later emphasized, made an ideal arrangement for internal secretion.

In 1874, Born wrote his paper on the development of the horse ovary, and, although handicapped through lack of fetal cases (one 10-month and one term fetus), he left little to be added from a morphological viewpoint. He quotes Franz Müller as having stated (1871) that the ovaries of a 7- to 8-month horse fetus were much larger than those of a newborn foal, and Chauveau that the fetal horse ovary was larger than that of a grown horse. Born noticed a resemblance between the interstitial cells of both the testis and the ovary (his Keimlager cell) and luteal and hepatic cells.

Tourneux (1879) studied the gonads of two female and three male horse fetuses and compared the interstitial cells of the testis with those of the ovary. Born had already suggested the analogy.

Plato (1896-1897) described the pigmented interstitial cells of the horse testis and thought that their lipochrome granules penetrated the Sertoli cells to nourish the sperm.

Bouin and Ancel ('03) reviewed the situation of the interstitial cell, paying most attention to the male gonad, and emphasized the concept of an interstitial gland, the organ of secretion of the sex hormone. Bouin and Ancel ('04) like Limon ('02), studied the interstitial cell in most available species, including the horse. In the testes of cryptorchid horses they observed a massive hyperplasia of the interstitial cells and degeneration of the seminiferous tubules. Such animals exhibited all the sexual vigor and secondary sex characteristics of a normal horse. This observation was also made by Whitehead ('08), who contributed a histological study on the testes of ridgelings and mules.

In 1907, Aimé investigated the interstitial cell of the ovary in various forms (pig, sheep, horse, man, goat, dog, rabbit) and gave a very detailed description of the growth and degeneration of that cell, as observed in the ovaries of eleven horse fetuses. He suggested that the internal secretion of these cells might cross the placental barrier and establish an equilibrium with the maternal luteal tissue.

Kohn ('26), in a well-illustrated paper reviewed the previous work on the development of the fetal horse ovary and gave a very complete account of its histology. He also described the single fetal horse testis in his series. Like Aimé, he sensed a significance of these hyperplastic gonads, but made no physiological contribution.

METHODS AND MATERIAL

The material used in this study was collected from range mares at various stages of gestation. These animals were being killed in a horse slaughter house. They originated on

the open range from close to the Mexican border in Arizona and New Mexico to as far north as Nevada and eastern Oregon. The weights of the animals varied, depending on breeding, the season of the year, and feed conditions on the range. The average mature weight of these mares was approximately 800 pounds and they represented the mustang type. In some shipments during the poor-feed season in the fall and winter practically all of the mares were in extremely poor physical condition. The gonads were removed from these fetuses as soon as the mares were eviscerated on the slaughter floor, weighed, the sex recorded and small pieces removed and placed in Bouin's fixative. In part of the specimens the gonads were not weighed, but measurements were taken of the length, width, and thickness. The remainder of the gonads were placed in 95 per cent alcohol, keeping the male and female glands in separate containers. At the same time crown-rump, in all cases, and some eye-ear measurements of the fetuses were recorded. Observations were made on the ovaries of the dams, and they confirmed our previous observations on the changes at various gestation periods. Samples of fetal blood were taken in some cases as well as other portions of the fetus, such as muscle and liver tissue. The blood was taken from the umbilical vessels in the larger fetuses and by severing the vessels in the neck of the smaller ones. The blood and tissues were also preserved in 95 per cent alcohol. The gonads from approximately 300 fetuses have been examined.

DEVELOPMENT OF FETAL GONADS

In presenting our data on the fetal horse gonads, we will give particular attention to the correlation of the development of the fetal gonads with the condition of the maternal ovaries and with the hormonal concentrations in the mare. In view of the considerable work of earlier investigators on the histology of the fetal gonads, we will limit our presentation to what we feel are the salient facts.

The gonads of the horse undergo a rather remarkable growth during intra-uterine life, the fetal gonads usually exceeding those of the maternal ovaries in weight when the fetus has a crown-rump length of 50 to 60 cm. The weights of the gonads taken from fetuses in various stages of development are given in figure 1. The weights recorded include both gonads of each fetus. The development of the fetal gonads runs parallel in both sexes (fig. 7). The gonads are very small in fetuses having a crown-rump measurement of

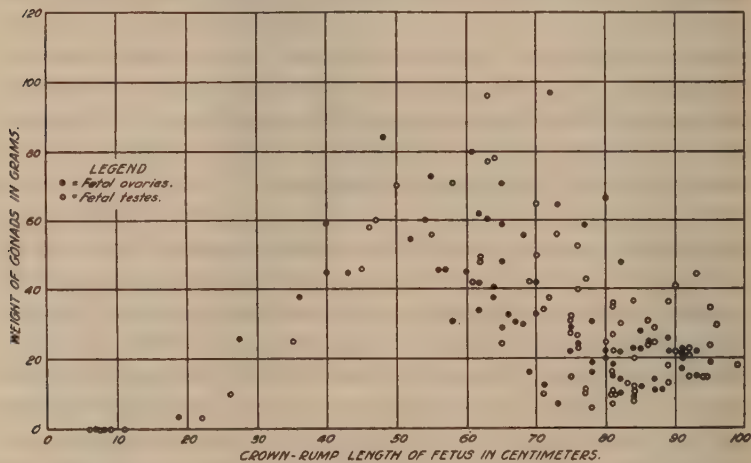


Fig. 1 Graph showing the relation between the weights of the fetal gonads and the C. R. length of the fetus. The weights refer to both gonads of each fetus.

7 cm. and were placed on the base line in figure 1 because they weigh under 1 gm. (ovaries of fetus 35, for example, weighed 140 mg.).

The fetal ovaries during the major portion of the pregnancy period consist of an almost solid mass of interstitial cells. These interstitial cells are present in very young fetuses and first appear singly or in small island-like clumps distributed throughout the medullary portion of the gonad (fig. 8). Figure 6 shows these interstitial cells at a higher magnification. We have observed this arrangement of the

interstitial cells in the gonads of fetuses ranging in crown-rump length from 4 to 12 cm. Thus the interstitial cells appear sometime previous to the 4 cm. stages. At this early stage (4 to 12 cm.) the cortex of the ovary shows cords of germ cells interspersed in a connective tissue framework. A cross section of an ovary of a fetus with a crown-rump length of 7 cm. together with a cross section of the maternal ovary, is shown in figure 2. At this stage a luteinizing process is taking place in the maternal ovaries, due to the high concentration of the gonad-stimulating hormone in the blood serum of the mare.

At the same stage (4 to 12 cm.) the structure of the fetal testis shows islands of interstitial cells morphologically similar to those found in the ovary. There are also tubes of sex cells similar to those found in the cortex of the fetal ovary which are, however, distributed throughout the body of the testis (fig. 13).

The gonad-stimulating substance disappears from the blood of the mare when the fetus has attained a crown-rump measurement of about 30 cm. The maternal ovary has now passed the stage of greatest development of the corpora. Figure 3 shows the maternal and fetal ovaries at the time when the former are at the height of their development. At this time (12 to 25 cm. C.R. length) the interstitial cells in the fetal ovary formerly present in islands have become a solid mass. The germ cells comprising the cords at the periphery have become buried in a dense fibrous cortex less than 1 mm. in width and show no evidence of dipping into the interstitial cell mass making up the medullary portion of the ovary. In the testis at this stage the tunica albuginea is devoid of cords of cells and has become a well-marked connective tissue membrane (fig. 15). The germinal epithelium is no longer present on the surface of the testis. Tubules of sex cells are sparsely scattered throughout the interstitial cell overgrowth.

From this time on the fetal gonads continue to increase in size coincident with regression in the maternal ovaries. The

maximum size of the fetal gonads is reached when the crown-rump measurement is from 45 to 65 cm., at which time the maternal ovaries are devoid of lutein tissue and have apparently become inactive (fig. 4). The enlargement of the fetal gonads is for the most part the result of an increase in the number and size of the interstitial cells. Sections of the fetal ovaries show the interstitial cells to be closely packed together with a large amount of cytoplasm surrounding the nuclei and giving evidence of actively secreting cells (fig. 9).² The fetal gonads of a number of other species have been investigated, as, for example, by Simpkins ('28) and Felix ('12) in man; Allen ('05) in pig and rabbit; Bascom ('23) in cattle, but in none of these species is there any indication of a comparable interstitial cell overgrowth. The cortex of the ovary at this stage has widened and still contains many cords of germ cells which are poor in cytoplasm and which show evidence of regressive changes. The fetal testis at this stage (45 to 65 cm. C.R. length) likewise consists of an almost solid mass of interstitial cells among which are sparsely scattered developing seminiferous tubules as mentioned above for the earlier stage.

After the maximum stage of development has been reached, there is regression, and in some cases gonads taken close to the time of parturition weigh approximately $\frac{1}{10}$ of those taken at maximum size. The inactive maternal ovaries may exceed the fetal ovaries in size at this time (fig. 5). Figure 1 shows that there is a great variation in size of the gonads from fetuses having the same crown-rump measurement, for example, at 80 cm. length. This variation, observed in later stages of pregnancy, is undoubtedly caused by two or possibly three factors. First, the crown-rump measurement is not a very accurate index of the age of the fetus, because all fetuses having the same period of intra-uterine life are not the same size. Also, there is great variation in the length

² We have some evidence that, coincidentally with the development to maximum size of the fetal gonads, there is an increase in the amount of oestrin in the urine of the mare.

of the gestation period of the mare. Tessier (1825) stated that in 582 mares impregnated after one service the shortest period of gestation was 278 days and the longest 419 days—a difference of 132 days. In 312 mares which were bred several times the shortest period of gestation calculated from the last service was 290 days, and the longest 377 days—a difference of 87 days. The third possible factor is the supposition that the period of time during which regression of the gonads occurs may vary in different fetuses and may be involved in environmental factors such as nutrition.

Early evidence of break-down in the interstitial cells in the fetal gonads is seen in the cytoplasmic deposition of a lipochrome which is not dissolved out by dehydration with alcohol or treatment with xylol. Later, there is a shrinkage in the size of most of the interstitial cells and their nuclei become pycnotic and degenerate completely (compare figs. 11 and 12). These degenerative changes are the main cause of the reduction in size of the fetal gonad in late intra-uterine life. Some of these cells may persist and enter into the composition of the theca interna of small follicles which now appear in the peripheral zone of the interstitial cell mass (fig. 10).³ There are cords of sex cells arising from the germinal epithelium penetrating into the dense fibrous cortex. The fetal testis at this stage also shows massive fatty degeneration of the interstitial cells. As in the ovary, some of these cells completely degenerate. Some seem to revert to a fibroblast-like type (fig. 14). Others continue further into post-fetal life.

The tremendous development of the fetal gonads occurs after the disappearance of the gonad-stimulating hormone from the maternal blood and after the regressive changes have progressed in the maternal gonads. The great development of the maternal ovaries is undoubtedly due to the presence of the gonad-stimulating hormone in the blood stream. The stimulus for the formation of the interstitial cell mass in the fetal gonads is less apparent. This cell can be seen

³ Some of these interstitial cells may still be seen in the ovary of the 2-year-old filly.

in fetal ovaries taken at the time of maximum concentration of the gonad-stimulating hormone in the maternal blood. The fetal gonads reach their maximum development, however, after the gonad-stimulating hormone is no longer detectable in the maternal blood stream.

HORMONAL CONTENT OF FETAL HORSE GONADS

We are not aware that any of the previous workers with fetal horse gonads have tested these tissues for hormonal content. Koch ('31) found fetal bull testicular extracts more potent for the male sex hormone than those made from the mature gonads.

Such quantities of almost pure gonadal interstitial tissue for extractive purposes probably can not be found anywhere else in such abundance as in the fetal gonads of the horse. In the ovary the germ cell plaque may be sliced off the surface of the ovary and the remaining mass (often weighing over 50 gm.) of pure interstitial tissue extracted. In the testis the proportion of germinal tubules to interstitial tissue may be seen in figure 15. We have tested these tissues for the presence of progestin, oestrin, and male sex hormone.

Aimé ('07) suggested that the secretion of the fetal gonads might be supplementing that of the maternal corpus luteum. In order to check this hypothesis, the fetal gonads were extracted for progestin (corporin) by the methods of Allen ('30) and Fevold, Hisaw, and Leonard ('32). The tests for progestin were negative in all instances. A total of eight tests was made. Extracts of fetal blood were used in three of these, extracts of fetal ovarian tissue in four, and extract of fetal testes in one. The following tests will serve as examples:

Three hundred grams of fetal horse ovaries extracted by Fevold, Hisaw, and Leonard's ('32) rapid method up to the ethyl ether stage yielded 3.12 gm. of a thick, brownish, oily material. Of this a total of 2.22 gm., in four daily doses was given subcutaneously to virgin rabbit W16, spayed on the day injections were begun. Autopsy on the fifth day revealed

extensive uterine and vaginal hemorrhage, the mammary areas greatly congested, and enlarged oedematous uterine horns. Microscopic examination showed a strong oestrin reaction, but no progestational proliferation.

Three hundred grams of fetal horse testes treated in like manner yielded 2.1 gm. of extract—a total of 1.3 gm. of which was given in four daily doses to spayed virgin rabbit W17. Again a strong oestrin effect was obtained, but no progestational proliferation.

Inasmuch as oestrin reactions were obtained in rabbit uteri when the extracts used for the progestin tests were injected, a number of fetal tissues were extracted by the methods cited above and tested for oestrin in spayed rats. In addition, we found that when the tissues were extracted for male sex hormone after the method of Koch and Gallagher ('31), these extracts likewise gave oestrin reactions when injected into spayed rats.

The following tissues taken from fetuses ranging in crown-rump length from 50 to 95 cm. were extracted: blood, liver, ovaries, and testes. Most of the gonadal tissue tested consisted of aggregate samples from several fetuses and thus no effort will be made at this time to show relative concentrations at different stages of development. Further, different tissues from the same fetus have not been compared in a sufficient number of cases to allow us to speak on the relative concentrations in the various tissues.

Extracts of fetal blood, liver, ovaries, and testes obtained by any one of the methods used gave oestrin reactions when injected into spayed rats (table 1). We have found that much smaller quantities are necessary when the extracts are smeared into the vaginas of spayed rats. It will be noted that as small an amount as 0.15 gm. of fetal ovaries or fetal testes gave a response when the extract was administered by the latter method. It took 8 to 32 gm. of these tissues when the extracts were injected subcutaneously. This great discrepancy in the two methods of administration is possibly explained by the fact that there was very poor absorption of our extracts when injected beneath the skin. All tissues ex-

amined have shown a rather uniform concentration of this hormone with the extraction methods used. The testing of several tissues from the same fetus, however, may give very different results. At this point we would like to again men-

TABLE 1
Tests for oestrin in fetal horse tissues

TISSUE USED	METHOD OF EXTRACTION	METHOD OF ADMINISTRATION TO SPAYED RATS	DOSE GIVEN IN TERMS OF FRESH TISSUE	NUMBER OF RATS USED AT EACH DOSAGE LEVEL	NUMBER OF RATS GIVING POSITIVE VAGINAL REACTIONS
			<i>Grams</i>		
Fetal ovaries	Fevold, Hisaw, and Leonard ('32)	Smeared into vagina	0.1	6	6
Fetal ovaries	Koch and Gallagher ('31)	Smeared into vagina	0.15	4	4
Fetal ovaries	Allen ('30)	Injected sub-cutaneously	16.0 8.0 4.0	1 1 1	1 None None
Fetal ovaries	Allen ('30)	Injected sub-cutaneously	16.0 8.0 4.0	2 1 1	2 None None
Fetal ovaries	Fevold, Hisaw, and Leonard ('32)	Injected sub-cutaneously	32.0 8.0 2.0 0.5	2 2 2 2	2 1 None None
Fetal testes	Fevold, Hisaw, and Leonard ('32)	Smeared into vagina	0.14	6	6
Fetal testes	Koch and Gallagher ('31)	Smeared into vagina	0.15	4	4
Fetal testes	Fevold, Hisaw, and Leonard ('32)	Injected sub-cutaneously	32.0 8.0 2.0	2 2 2	2 None None
Fetal liver	Fevold, Hisaw, and Leonard ('32)	Injected sub-cutaneously	32.0 8.0 2.0 0.5	2 2 2 2	2 2 None None
Fetal blood	Allen ('30)	Injected sub-cutaneously	27.0 9.0 3.0	2 2 2	None None None
Fetal blood	Allen ('30)	Injected sub-cutaneously	50.0 20.0	2 2	2 None

tion that about 30 cc. of maternal blood serum taken during corresponding stages of pregnancy must be used to produce the oestrin reaction in spayed rats, whereas $\frac{1}{50}$ cc. of urine will give the same response. The presence of oestrin in the various fetal tissues makes it necessary to consider the fetus as one possible source of the large amounts of oestrin excreted in the urine.

The extracts prepared after the Koch and Gallagher ('31) method up to the hexane-70 per cent alcohol step were also tested for the presence of male sex hormone, using the method suggested by Korenchevsky ('32) on immature castrated rats. The guinea pig seminal vesicle prostate test was also made. Extracts made from ovaries, testis, and corresponding livers were each tested. The prostate and seminal vesicles of animals receiving ovary and testis extracts were from 50 to 100 per cent heavier than those of the controls. The liver extracts produced very little change. The presence of the male sex hormone was therefore not definitely demonstrated.

SUMMARY

The fetal gonads of the horse during the major portion of intra-uterine life, consist of an almost pure interstitial cell mass. This interstitial cell mass reaches its maximum size after the gonad-stimulating hormone is no longer demonstrable in the maternal blood stream. We have demonstrated the presence of oestrin in various fetal tissues. We suggest the possibility that the large amounts of oestrin excreted by the mare in the urine during the second half of pregnancy, when the maternal ovaries are quiescent, may be of fetal origin.

ADDENDUM

Data, accumulated while this article was in press, suggest that the oestrin present in fetal tissues and mare urine is secreted by the placenta. Also, it has been shown, by some modifications in the method of preparing the extracts to be injected subcutaneously, that the oestrin concentration in fetal tissues is somewhat higher than is indicated in table 1.

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PLATE 1

EXPLANATION OF FIGURES

2 Unstained cross sections of maternal (m) and fetal (f) ovaries (C. R. length of fetus, 7 cm.). The maternal ovary contains two corpora (cor.) and two small follicles (fol.). The histological character of the fetal ovary at this time corresponds to that shown in figure 8. $\times \frac{1}{4}$.

3 Unstained cross sections of maternal (m) and fetal (f) ovaries (C. R. length of fetus, 22 cm.). Four almost solid corpora (cor.) are present in the maternal ovary. $\times \frac{1}{4}$.

4 Unstained cross sections of maternal (m) and fetal (f) ovaries (C. R. length of fetus, 50 cm.). The maternal ovary has shrunk to a small fraction of its previous size (compare with figure 3) and only scattered pigmental remnants (p) of the corpora remain. During the period when the maternal ovary regresses, the fetal ovary develops rapidly and now consists of an almost solid mass of interstitial cells enclosed in a fibrous cortex less than 1 mm. in width. $\times \frac{1}{4}$.

5 Unstained cross sections of maternal (m) and fetal (f) ovaries near term (C. R. length of fetus, 85 cm.). The fetal ovary has shrunk to about the size of the inactive maternal ovary. $\times \frac{1}{4}$.

6 Photomicrograph, showing the interstitial cells seen in figure 8 at high magnification. By comparing with figure 9, it can be seen that the stroma becomes less conspicuous as development proceeds. $\times 300$.

7 Photographs of male and female gonads. The figures in connection with each gonad refer to the crown-rump length of the fetus in centimeters. The male gonads are distinguished from the female gonads at an early date by the vascular network on the surface of the former. $\times \frac{7}{15}$.

8 Photomicrograph, showing the sex cords in the cortical area and the islands of interstitial cells in the medullary portion of a fetal ovary (C. R. length of fetus, 12 cm.). $\times 70$.

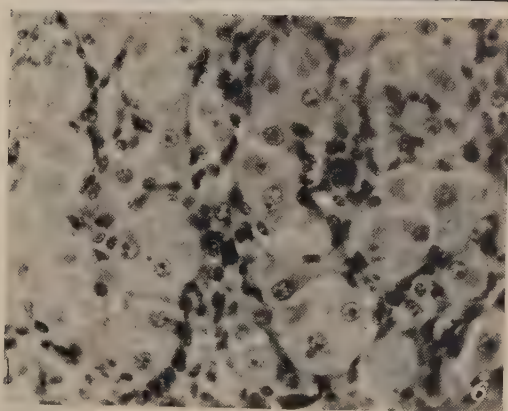
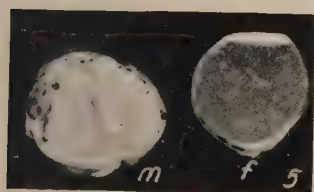
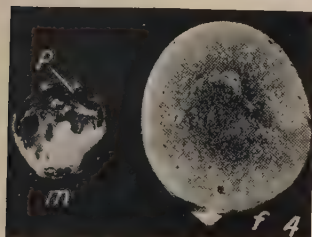
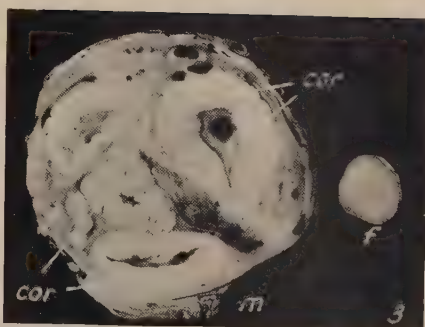
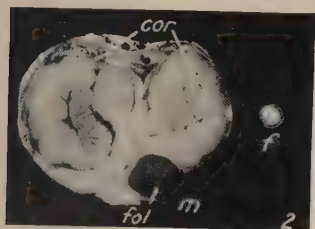


PLATE 2

EXPLANATION OF FIGURES

All figures are photomicrographs.

9 Interstitial cells in the ovary of a fetus with a crown-rump length of 50 cm. This is a section of the ovary shown in figure 4 and represents the character of the entire ovary, excepting for the thin fibrous tunica. $\times 225$.

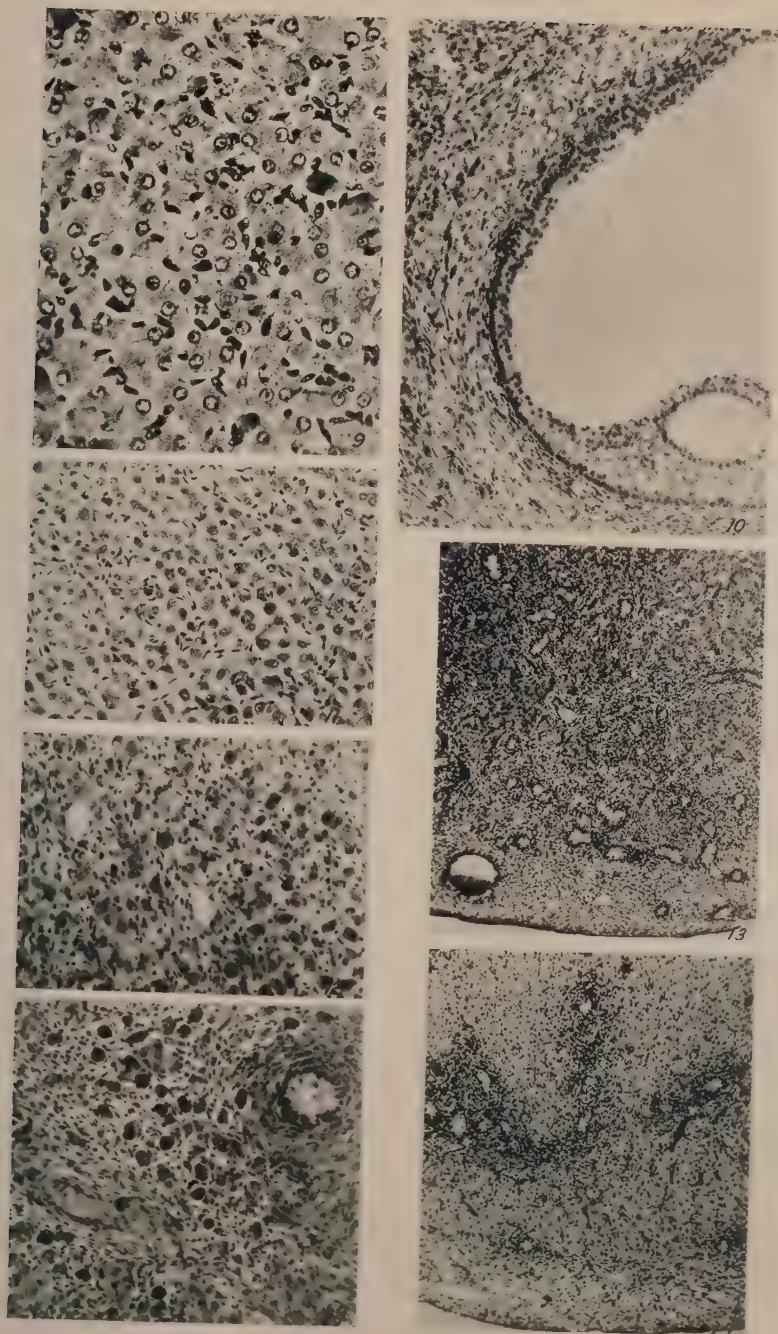
10 Near term follicles begin to appear in the peripheral zone of the interstitial cell mass (C. R. length of fetus, 95 cm.). $\times 130$.

11 and 12 Cross sections of fetal ovaries from a 65-cm. C. R. length fetus and from a 95-cm. C. R. length fetus respectively, taken at approximately the same magnification, to show the decrease in number of interstitial cells and the increase in stroma in later intra-uterine life. $\times 130$ and 150 .

13 Fetal testes (C. R. length of fetus, 12 cm.). The sex cords are distributed throughout the testes. Interstitial cells, although present, are fewer in number than in later stages (compare with fig. 15). $\times 50$.

14 Fetal testis at term (C. R. length of fetus, 95 cm.). As in the ovary, the interstitial cells are reduced in number prior to birth. Most of those present are in late stages of 'fatty degeneration.' $\times 150$.

15 Fetal testis (C. R. length of fetus, 16 cm.). The interstitial cells have become closely packed between the scattered developing seminiferous tubules. $\times 50$.



STUDIES IN MICRURGICAL TECHNIQUE

V. A MOIST CHAMBER FOR TISSUE CULTURE AND CELLULAR DISSECTION

J. ARTHUR REYNIERS

Department of Biology, University of Notre Dame, Notre Dame, Indiana

THREE FIGURES

Moist chambers employed in micrurgical procedures generally take the form of a box, small enough to fit on the stage of a microscope. They are usually constructed of glass microscope slides, lined with moistened blotting paper, and roofed with a cover glass, the underside of which acts as a working surface. These chambers are used to prevent physical changes in small bits of tissue or in single isolated cells. They can be used in experimental investigations involving the dissection of single cells, the culturing of tissues or physical and physiological experiments on living protoplasm.

The usual type of moist chamber in use at present is satisfactory for simple micrurgical procedures, but it lacks certain mechanical refinements, the addition of which, would permit its more successful use in experiments that are carried over longer periods of time when experimental and environmental conditions of moisture, temperature, and the like must remain constant. Most moist chambers, for instance, will not allow a constant gaseous tension, a controlled temperature and uniform moisture conditions to be maintained during the actual manipulation of the specimen. This can be achieved only when the moist chamber is entirely enclosed, even during manipulation, and when the enclosure is incorporated as part of the design and not merely an accessory to it.

The moist chamber presented in this paper has these advantages in that it is entirely enclosed during work, a constant gaseous tension can be built up and maintained, it can be used with any micro-manipulator, it is electrically heated to a constant temperature which is controlled with a thermostat, pipettes may be changed without exposing the interior of the chamber, it is adaptable to any microscope, and by using replaceable moist chambers any number of experiments may be carried out simultaneously with the same apparatus.

ACKNOWLEDGMENT

Acknowledgment is made of the assistance received from Dr. Francis J. Wenninger and Dr. Theodore Just, of the University of Notre Dame, in the preparation of this work and in their criticisms of the results. My father, Mr. Leo A. Reyniers, built the apparatus described herein.

DESCRIPTION OF THE WRITER'S APPARATUS

The apparatus described in this paper works on a cross-slide principle. It consists of four main parts: 1) the housing in which a moist chamber cradle is moved; 2) the moist chamber cradle; 3) the removable moist box in which the actual work is performed; 4) the stage upon which the apparatus is manipulated.

1. The cradle housing (fig. 2, dia. B)

The housing in which the moist chamber cradle is moved is built in the form of a frame. Its inner dimensions are three times as wide as the cradle, but exactly as long. The ends of the housing are built double, the thicker of the two pieces is $\frac{1}{4}$ inch less wide than the housing and rests against a thinner partition. The thicker end piece can be moved back and forth by means of a lead screw anchored in the side wall. In order that this movement be in a straight line, two guide pins are fastened on each side of the moving end piece. The side walls are built double so that heating elements, wound

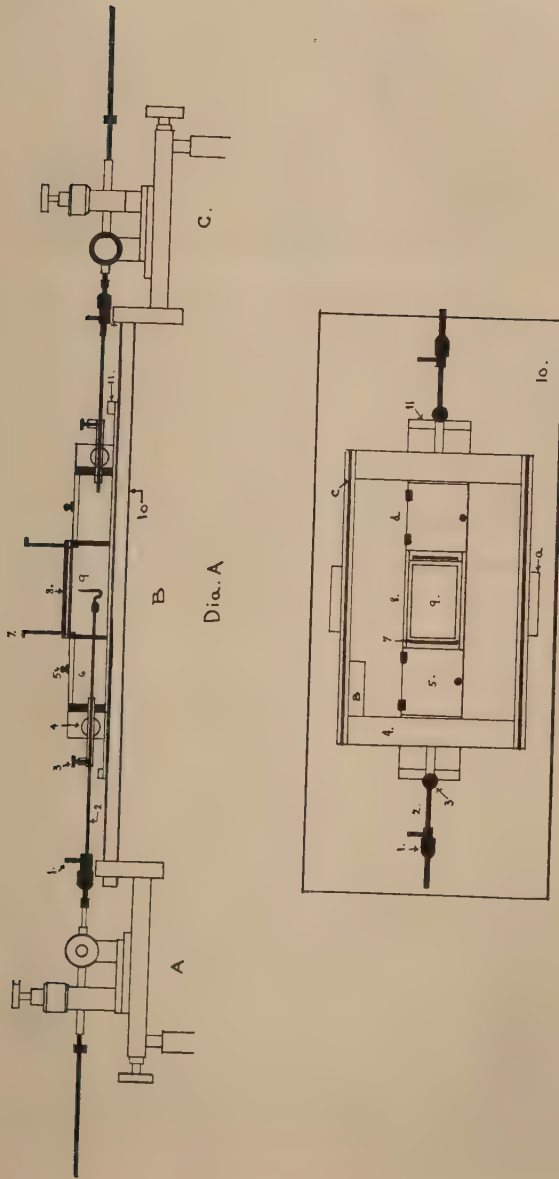


Fig. 1 Diagram of moist chamber. A, side view. B, top view. Dia. A Side view of chamber. 1, swivel joint; 2, pipette holder; 3, guide pin; 4, ball acting as a pivot for the pipette system; 5, trap door over pipette changing compartment; 6, pipette changing compartment; 7, sliding door to close off moist chamber; 8, frame for fastening down the working surface; 9, moist box; 10, stage upon which the moist chamber is moved; 11, extensions from moist chamber housing. A, micro-manipulator; B, moist chamber; C, micro manipulator. Dia. B Top view of moist chamber. Numbers correspond to those given in diagram A. a, guide which fits into a slot cut into the cradle wall; b, adjustable thermostat; c, heating units; d, hinges.

on mica strips, can be placed between them. The side wall of the cradle corresponding to the wall of the moist chamber which bears the thermometer and gas pipes has holes cut into it to accommodate these features. A thermostat is mounted on the inner wall of the housing. This thermostat is 2.75 inches long, 1.25 inches wide, and 0.275 inch thick, is adjustable by a screw, and is commercially used at present on electrically heated incubation chambers or section warming apparatus.

The ends of the housing are constructed to take the pipette system. This system consists of a square metal tube called a pipette holder; a sleeve through which the pipette holder passes, an adaptor for the pipettes, and a ball through which the system is moved. This system is double, i.e., there is one such system on each end of the housing. The ball which is used as an axis for the pipette system is centered in the end wall and is held in place between a split socket bearing. A pipette holder sleeve made from brass tubing is fitted through the ball and passes through an elongated slot in the end wall.

2. Moist chamber cradle (fig. 2, dia. C)

The moist chamber cradle carries a moist chamber and two changing compartments. It is built to fit accurately inside the housing walls, so that while it can be freely moved back and forth, it still remains air-tight. Windows are cut in the ends of the cradle to allow for pipette manipulation. Little doors are hinged on top of the end walls at either end of the cradle. The floor is covered with a brass strip exactly as wide as the cradle, but 1.5 inches long at each end. The extensions so formed pass under the end walls of the housing and are secured in the arms of a mechanical stage. The center part of the floor is cut out to admit light.

3. The moist box (fig. 2, dia. D)

The moist box is made of two cylinders, one fitting inside the other. The inner cylinder is slightly longer than the outer cylinder and when in the cradle is held stationary by a small

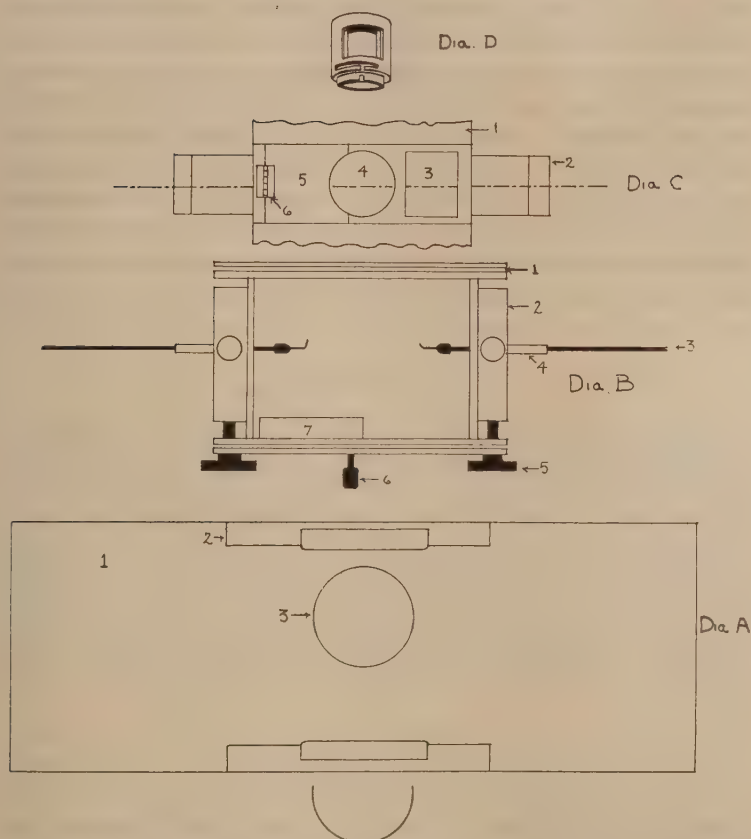


Fig. 2 Diagrammatic detail of moist chamber. Dia. A Top view of stage upon which the moist chamber is operated. Dia. B The cradle housing. Dia. C Top view of moist chamber cradle. Dia. D Replaceable moist chamber. Dia. A 1, stage; 2, tongued guide; 3, hole to admit light. Dia. B 1, double side walls between which the heating units are fastened; 2, movable end wall; 3, pipette holder; 4, sleeve; 5, lead screw to adjust the pipette tip; 6, control screw for thermostat; 7, thermostat. Dia. C 1, wings to cover space between the moist chamber cradle and the housing; 2, extensions which are held in the arms of a mechanical stage; 3, pipette changing chamber with trap closing door removed; 4, moist chamber socket; 5, trap door for closing the changing chamber; 6, hinge.

stud. Two openings, opposite one another, are cut through the walls of both cylinders. When the outer cylinder is turned, the openings are closed. In order to guide the outer cylinder as it is turned to close up the openings, a stud is fastened, through a slot in its wall, to the inner cylinder. The floor of the moist box can be covered with mica, which is held in place by a frame, the roof will, of course, form the working surface. This form of moist box is very convenient to use and a number of such chambers can be constructed to fit the same instrument so that they may be incubated apart from the micro-manipulator and so permit several experiments to be carried out simultaneously.

4. The stage of the microscope (fig. 2, dia. A)

The moist chamber is manipulated on a special microscope stage made of brass. The size of the stage depends on the size of the moist chamber which is, in turn, limited by the nature of the work. For convenience the stage is built to replace the one which comes with the microscope. The moist chamber is moved from side to side on the microscope stage by a mechanical stage. The housing is guided in a straight line by two guide bars. These bars are tongued and fit into slots milled along the outer walls of the housing.

Heating the moist chamber

The moist chamber is heated with coils of chromel wire wound on mica strips. These coils are located between the double side walls of the housing. Carbon filament electric light bulbs serve as a resistance between the line connection and the heating elements and act as a pilot light to indicate when the current is turned on. The thermoregulator is of the usual bi-metal type and is regulated by a screw point. Temperatures between 30°C. and 100°C. can be regulated for a variation of less than 1°, depending on the size of the chamber and the quality of its insulation.

In order to conserve heat, a shield is made to fit over the space between the moist chamber cradle and its housing. This shield consists of two metal wings which are fastened to the side walls of the cradle. With this arrangement the moist chamber can be moved to any position without allowing a gap to appear between it and the housing.

The outside walls of the cradle, the upper part of the shields, and the inner surface of the microscope stage are insulated with strips of thin felt over which equally thin strips of linoleum may be glued. Because the thermometer is within the pipette changing chambers, the temperature of the moist chamber can be regulated quite accurately.

The pipette system

The pipette system consists of a pipette holder, a pipette adaptor, a ball and socket joint, and the micro-manipulator.

The pipette holder is made from a piece of brass tubing which is threaded on both ends. This holder fits into a sleeve which has been mounted through a ball fitted into the end wall of the cradle. A pin mounted into one end of the pipette holder sleeve prevents the pipette tip from turning. The pipette holder may also be made from square brass tubing and fitted into a sleeve which has been drawn over a square arbor.

The ball and socket joint by which the moist chamber is connected to a micro-manipulator must be carefully made. This joint is illustrated in diagram A, figure 3. Any type of micro-manipulator may be used with this apparatus, although the manipulator described in a previous publication (Reyniers, '32) is more convenient to use.

Pipettes or micro rods of glass are made on a mechanical pipette puller described in the third article of this series (Reyniers, Micrurgy III, '33). Pipettes used in this machine are comparatively short to those more generally used. They are mounted in an adaptor which screws onto the end of the pipette holder. These adaptors are made by bending a piece of 18-gauge hypodermic wire into a modified L form. The

short arm of the L is bent not quite to right angles, nor is it exactly in line with the shaft. A smaller piece of 27-gauge hypodermic wire is soldered into the shorter arm so that it projects $\frac{1}{16}$ of an inch. The long arm of the L is soldered into an adaptor base which is simply a short piece of brass tubing threaded on the inside to fit the end of the pipette holder.

Pipettes are made from capillary tubing which will fit over the adaptor base. They are fixed in place by first touching

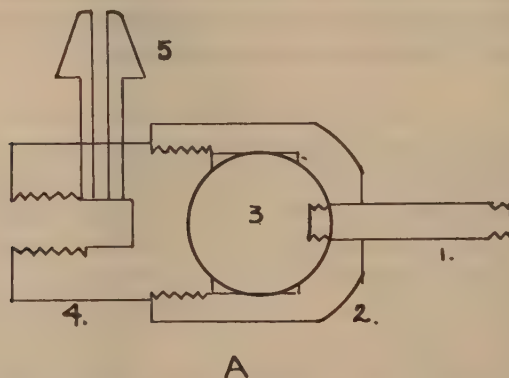


Fig. 3 Detail of swivel joint for connecting the moist chamber with a micro-manipulator. 1, connection to manipulator; 2, screw cap; 3, ball; 4, connection to moist chamber; 5, air connection.

a bit of vaselin or paraffin to the finer adaptor needle; warming the base of the pipette and finally pushing it into place. The melted substance at the base of the fine needle runs up into the pipette base for a short distance and forms an effectual seal.

USE OF THE MOIST CHAMBER

Moisture is supplied to the chamber from blotting paper pads. As the moist box is always closed and held to a constant temperature, little difficulty is encountered in saturating it.

The swivel at the end of a pipette holder is fastened into the end of the pipette holder which is a part of the micro-manipulators set into place at each end of the apparatus. A working surface is fixed in place on the moist chamber, the chamber is closed off, the electric current is turned on, and the temperature regulated. When the proper temperature has been attained, the moisture conditions within the chamber should become rapidly stabilized. During the interval when the chamber is being stabilized several micro-pipettes are made on the mechanical pipette puller.

Assuming that a micro-manipulator of the type described in a previous paper (Reyniers, '32) is used, the pipette holders on both sides of the moist chamber are pulled back until the pipette adaptors are in the changing compartments on each side of the moist chamber housing. The moist box is closed, and the doors over the pipette changing chambers are opened. A pipette adaptor, held in a pair of forceps, is screwed into place by turning the pipette holder. Pipettes are sealed in place on the adaptors, the pipette tips are lowered and the trap doors over the changing compartments are closed. The moist box is opened and the pipette tips are pushed to the center of a low power field. After centering the pipette tip the set collars on the pipette holder, which are a part of the micro-manipulators, are screwed tight. The tip is further centered in a high power field by use of the micro-manipulators.

When a pipette is changed, it must first be returned to the center of the field and then lowered, after which it is simply pulled back until it is in the pipette changing compartment. A new pipette may be mounted on the adaptor and it may be returned to the field by pushing the pipette holder forward until it is checked by the previously adjusted set collar.

Three distinct movement mechanisms are used in this apparatus, 1) to center the pipette tip, 2) to manipulate the pipette tip, 3) to expose different areas of the working surface. The pipette tip may be centered from front to back in the field by means of the lead screw in the side wall of the

housing. It may be centered from side to side by pushing the pipette holder forward until it is checked and completing this adjustment with the manipulator. After centering the pipette tip, it may be manipulated in the field by the movements on the micro-manipulator. Different areas of the working surface are exposed by using the mechanical stage to move the moist chamber. A movement of the stage from side to side moves the housing which carries the moist chamber with it. A movement of the mechanical stage from front to back moves only the moist chamber cradle and allows the housing to remain stationary.

It is well to bear in mind that before any adjustments are made in the moist chamber the pipette holders bearing the pipettes must be returned to neutral, i.e., in a straight line with respect to the housing and micro-manipulator. If this detail is observed the pipette tip is unaffected by any movement of the apparatus other than movements imparted to it by the micro-manipulators.

SUMMARY

The moist chamber whose construction is described, involves a new application of the cross slide, floating center principles. The chamber may be kept closed during work in which the experiment is carried over long periods of time. This allows for a minimum of change in the physical conditions within the chamber. Gaseous pressures may be built up and maintained at will. The chamber may be heated to any desired temperature and will maintain itself automatically at this temperature. This apparatus can be used with most micro-manipulators now in use or it can be worked by hand. It can be constructed larger or smaller to be worked under high powers or beneath the lower powers of a dissecting scope, the sweep of the pipette being adequate under any power. The pipette is moved in an arc movement, but a moist chamber has been constructed on this principle so that the pipette tip is moved in straight lines (Reyniers, *Micrurgy* II, '33). Specimens may be worked on the underside of a cover slip or

they may be worked on the floor of the chamber if it is built slightly lower.

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STUDIES IN MICRURGICAL TECHNIQUE

VI. A MICRO SUCTION AND INJECTION APPARATUS

J. ARTHUR REYNIERS

Department of Biology, University of Notre Dame, Notre Dame, Indiana

TWO FIGURES

Micro-pipettes are instruments used to inject by pressure minute quantities of fluid, or to collect by suction small particles such as might be found in the interior of a protozoan cell. In single cell isolation such instruments are used to form or collect micro-droplets in which the bacterial cell is isolated. Work of this nature requires an accurate control of the forces used, whether they be the forces produced by expansion or contraction of mercury or air, the force produced by driving a piston against an enclosed volume of fluid, or the force produced by compressing the walls of a cylinder.

In droplet formation, no matter what system is used to create the droplets, a certain amount of difficulty is experienced with the built-up pressure which remains in the system after the droplet has been formed. Furthermore, difficulty is experienced with the spurting effect which results from the ejection of fluid through a very small opening. Various methods have been devised to overcome these difficulties. The result has been many different types of apparatus, most of which employ a pusher of some sort to obtain the desired effect.

Micro-suction and injection pipettes have been devised by Barber ('11-'14), R. Chambers ('21-'22), T. Peterfi ('24), C. V. Taylor ('23-'25), R. L. Malone ('18). The Barber apparatus makes use of a pusher of mercury and depends on the contraction and expansion of this metal when it is heated

or cooled. R. Chambers ('21) describes an apparatus consisting of a thin-walled, steel cylinder filled with mercury. The driving force is produced by compressing the walls of the cylinder. T. Peterfi describes a high pressure vacuum pipette consisting of a simple valve system and a cylinder of gas under pressure. Vacuum is produced by a water pump. He also refers to a pipette in which he employs the physical principle of expansion of heated air and its contraction when cooled. C. V. Taylor's apparatus employs a mechanical means for varying the pressure on an enclosed volume of mercury. This system is both accurate and easily controlled. Malone uses a simple rubber nipple with a small hole cut in its top. Chambers ('22), advocates the use of a Luer syringe filled with oil or distilled water. Finally, in most single cell isolation work a simple mouth pipette is used.

The forces of surface tension, cohesion, evaporation, and the shape of the pipette, together with physical properties concerned with liquid pressure, are factors which must be considered before a successful instrument can be devised. 'Pusher' systems such as water filled systems or systems filled with mercury are difficult to use successfully because of the danger of diluting or contaminating the fluid which is injected. It would seem, then, that the ideal system should be one which is filled with air, in which the pressure can be built up slowly and mechanically and released instantly without danger of negative pressure. This system must fulfill general working requirements of routine and must not demand too much attention to manipulate. Finally, it would be desirable if some method of measuring the quantity of fluid injected could be incorporated in its construction. The system offered in this paper fulfills these requirements. It is essentially one in which air pressure can be built up slowly and released instantly thus offering absolute control of droplet formation or injection. A satisfactory experimental apparatus can be constructed with laboratory odds and ends and the apparatus can be built as large or as small as the range of pressure might require. Rather than describe the many

modifications of the system which has been used in these laboratories for work which varies from the making of micro-droplets on cover glasses, the injection of embryos and protozoan forms, to work where very high pressures are required, a simple laboratory model is offered which illustrates the principles involved.

DESCRIPTION OF THE APPARATUS

This apparatus is based on the principle of the compression of gases due to the weight of a column of mercury fed through a variable opening. Two glass columns are connected with glass tubing so that they take form of a U. One column which contains the mercury can be made from a burette; the other, or air compression chamber, is a simple glass tube larger in diameter than the burette. For the purpose of description the first tube is referred to as A, while the latter is designated as B. Tube A has a pear-shaped reservoir connected to its upper end. It is connected to tube B by the arms of a 3-way stop cock. Tube B is drawn out at its upper end so that a rubber tube can be easily slipped over the constricted end. This rubber tubing leads to a special push valve or a chemists' spring clamp and to the pipette holder. A small U-tube manometer may be connected ahead of the push valve.

The push valve used on this apparatus was designed to allow an immediate release of pressure by a single push on a button. While a chemists' spring clamp and a rubber tube will serve the same purpose and every bit as well, the push valve is more convenient to use and has the added advantage of being adjustable. The valve case is a block of steel. A hole is carefully drilled and reamed into this block and another smaller hole is drilled through the center of the block at right angles to the first. The plunger or valve stem is a simple steel rod of the same diameter as the larger of the holes drilled in the valve case. A groove is cut around this valve stem and the upper end of the stem is threaded for a short distance. The valve stem must be carefully lapped into place with diamond compound to prevent leakage of air. A

specially fitted collar is screwed onto the threaded end of the stem and a push button is fixed into place. The stem presses against a spring dropped into the bottom of the valve hole.

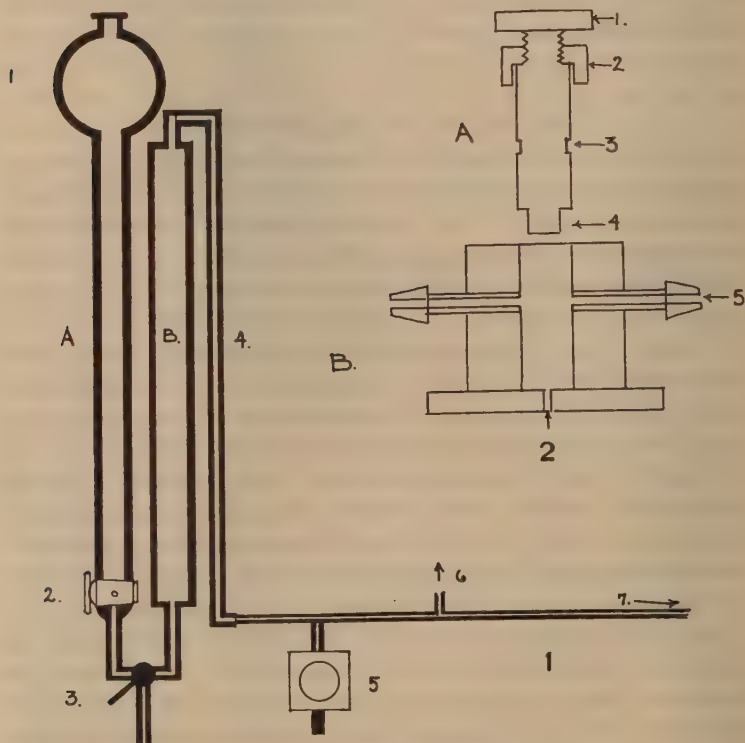


Fig. 1 Diagram of micro-pressure and suction apparatus. A, mercury tube; B, air column. 1, reservoir for mercury; 2, stop cock; 3, 3-way valve; 4, connecting tube; 5, push valve to release pressure; 6, connection to manometer; 7, connection to micro-pipette.

Fig. 2 Detail of push valve. A, valve stem; B, valve case. 1, push button; 2, adjustable stop to regulate valve opening; 3, shallow groove; 4, spring seat; 5, connecting pipes; 6, air vent.

To relieve the pressure which is built up against the bottom of the valve stem when it is compressed, a very small hole may be drilled from the bottom of the case into the hole in

which the stem fits. The valve may be easily regulated for opening size by moving the set collar. Once this collar is set, the valve is opened by a single push.

USE OF THE APPARATUS

Clean mercury is poured into tube A, until it fills the system up to the opening in tube B. During this filling process the release valve as well as the 3-way valve between tubes A and B are opened so that the passage is free from one tube to another.

If pressure is wanted, the operator must decide how much is required and how quickly it is to be applied. The valve in the bottom of the burette is opened accordingly. With the release valve closed, the pressure is allowed to build up evenly and may be watched on a manometer. The injection is followed under a microscope. As the pressure mounts, the fluid in the pipette starts slowly out toward the tip, or if it is already at the end, it starts slowly out into the object. As soon as the proper amount of fluid is injected, the pressure is released immediately by pressing the release valve. Since there is a return to atmospheric pressure in the system and no negative pressure is built up, the injection can be controlled very exactly. As soon as the injection is made, the flow of mercury is allowed to act continuously. When the air compression tube (tube B) is filled with mercury, it is emptied into a beaker through the 3-way valve. During this emptying process the release valve is to be left open.

If suction is wanted, tube B is allowed to fill while the release valve is opened. The mercury flow from column A is cut off, the release valve closed and the mercury in column A allowed to run out slowly through the 3-way valve into a beaker. When the desired amount of suction has been applied, the pressure is released. For ordinary work where only a few pressure operations and a correspondingly small number of suction operations are required, no special attention will have to be paid to filling and emptying column B, and only the 3-way valve needs attention.

If column B is graduated and the operator is familiar with the fluid to be injected as well as with the conditions of the injection, the operation of running up pressure is facilitated.

MODIFICATIONS OF THE APPARATUS

Many modifications of this apparatus have been built. These modifications are mentioned in this paper simply as suggestions to the operator, who will, no doubt, find some special refinement particularly adapted to his need and will himself modify the apparatus to that end.

A compact form of the apparatus has been built to use compressed air driven into a cylinder with a bicycle pump. The compressed air is used to drive mercury into an air compression chamber. An apparatus of this type can be mounted into a small box.

A small auxillary compression chamber has been connected into the air line so that a definite amount of pressure could be built up and released to kick out a small quantity of fluid from the pipette tip. This apparatus was used to form micro-droplets in single cell isolation experiments.

The apparatus described in this paper has been refined and specialized for the work at hand, but for all general purposes the simple apparatus made from laboratory odds and ends is satisfactory.

SUMMARY

A micro suction and injection apparatus is presented. This apparatus may be built from laboratory odds and ends. The principle employed is the compression of an air column by a regulated stream of mercury. The air compression can be built up as slowly as the operator wishes and may be instantly returned to normal by a check valve, thus effecting an immediate and absolute control of the suction or injection. Since pushers of mercury, water, and the like are done away with in this apparatus, there is little danger of contaminating or diluting the injected fluid or injuring the cell.

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